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Signal-dependent incorporation of MyoD-BAF60c into Brg1-based SWI/SNF chromatin-remodeling complex

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Transaction Report:

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

1st Editorial Decision

08 August 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two new referees whose comments are enclosed. As you will see, both referees express high interest in your manuscript and are in favour of publication, pending satisfactory experimental revision.

Specifically, the referees raise the following issues:

ref 1:

> In fig 4D why did Brg1 not associate with the myogenin promoter in differentiating cells expressing empty vector?

> Can you exclude that the N-terminus of MyoD associates with BAF60c, which could be a prediction from data in your previous Nature Gen paper.

The referee also raises a couple of further reaching recommendations, that would certainly make for a much more comprehensive dataset. While we would strongly encourage you to add such data in as far as it is available after a 2-3 month revision period, we will not make a comprehensive dataset to address the following two points a precondition for publication:

'It would be nice if the authors could extend this analysis to include other subunits of the Swi/Snf complex-to address whether BAF60c is indeed the only member of this complex to associate (with MyoD and/or the myogenin promoter) in proliferating myoblasts (or in cells treated with SB203580). ' and further 'it would be nice for the authors to evaluate whether the interaction between BAF60c and Brg1 is necessary to recruit other components of the Swi/Snf complex. One could do this by addressing whether knockdown of Brg1 reduces the association of other components of the Swi/Snf complex with the myogenin promoter in myocytes. '

ref 2

- > Could Brm be present at the promoter prior to differentiation? The referee recommends a directed ChIP experiment (including a positive control).
- > fig 2: need Western blot controls for RNAi.
- > fig 4: need Western blot controls.
- > fig5: add supporting data if available.
- > SI4: add data comparing Baf60 b and c directly.
- > SI6: ref 2 recommends removal, as the figure is not definitive.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Given the requirement for additional data, we will return the revision to one of the referees.

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, please visit our website: <http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of 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.

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

Prior work by Puri's group has established that p38alpha/beta activity is necessary to promote the association of Swi/Snf complexes with the myogenin promoter during skeletal muscle differentiation. In this work the authors extend this analysis to demonstrate that the C-terminus of MyoD associates with the Swi/Snf subunit BAF60c, and that both these components bind to the myogenin promoter in proliferating myoblasts. In addition, the authors present very compelling data that upon skeletal muscle differentiation, p38alpha/beta-mediated phosphorylation of BAF60C induces the association of the Swi/Snf component Brg1 to the myogenin promoter which induces subsequent chromatin remodeling and transcriptional activation. I think this is a great piece of work, which certainly merits publication in EMBO J. However, prior to publication, I highly recommend that the authors address the following issues:

1. The authors have very convincingly shown that BAF60c (but not Brg1) associates with the myogenin promoter in proliferating myoblasts. It would be nice if the authors could extend this analysis to include other subunits of the Swi/Snf complex-to address whether BAF60c is indeed the only member of this complex to associate (with MyoD and/or the myogenin promoter) in proliferating myoblasts (or in cells treated with SB203580).
2. The authors demonstrate that phosphorylation of BAF60c by p38alpha/beta is necessary to induce the interaction of BAF60c with Brg1 in vitro, and recruit Brg1 to the myogenin promoter in differentiated myocytes. However, along the same lines as point 1, it would be nice for the authors to evaluate whether the interaction between BAF60c and Brg1 is necessary to recruit other

components of the Swi/Snf complex. One could do this by addressing whether knockdown of Brg1 reduces the association of other components of the Swi/Snf complex with the myogenin promoter in myocytes.

3. In Figure 4D, I am surprised that Brg1 did not associate with the myogenin promoter in cells expressing empty vector and cultured in differentiation medium. The authors should comment on this point.

4. In a prior work (Nature Genetics, VOLUME 36 | NUMBER 7 | JULY 2004) Puri's group noted that co-transfection of Gal4-MyoD-N with either Brg1 or Brm boosted the ability of this construct to enhance the expression of a reporter gene. In light of the present data, the authors should address whether the N-terminus of MyoD similarly associates with BAF60c.

Referee #2

Forcales et al have submitted an outstanding and exhaustive body of work characterizing the molecular events that occur at the myogenin promoter prior to and following the onset of skeletal muscle differentiation. The work builds on a decade of work by numerous labs and ties together temporal and functional relationships between MyoD, histone acetylation, SWI/SNF chromatin remodeling enzymes and signaling through the p38 kinase pathway to generate a novel and extremely detailed model for the activation of myogenin in differentiating myoblasts. The work also identifies a specific role for a tissue restricted SWI/SNF subunit, Baf60c, that advances the general understanding about the diversity of subunit composition in SWI/SNF enzymes, a subject that has attracted significant attention in the last few years.

The reviewer received the somewhat unusual instruction from the Editor:
we are looking for authoritative referees who are prepared to assess the dataset in hand for its suitability for publication.

Therefore, if this truly is a "take it or leave it" decision, the reviewer casts an unequivocal vote for acceptance.

However, the reviewer still feels an obligation to provide the critiques that arose while reading the manuscript. There is one conceptual shortcoming that could only be addressed experimentally, but this point could be addressed in the future by the authors or others if the authors don't already have experimental evidence in hand to present in this manuscript. The other comments are relatively minor and likely can be addressed with text changes and/or additions or by addition of suggested controls.

The conceptual concern:

The authors' model suggests a MyoD-Baf60c complex on the myogenin promoter prior to myogenic differentiation and gene activation that is devoid of the Brg1 ATPase found in many SWI/SNF complexes. However, the authors do not account for the possibility that the related SWI/SNF ATPase, Brm, could be present at the promoter prior to differentiation. Although the authors indicate that they found no evidence for a stable association of Brm with MyoD in their protein interaction studies, this is negative data that could be influenced by experimental conditions. A more definitive experiment would be a directed ChIP experiment, though (i) a negative result would only be meaningful if there were a positive control showing Brm binding to another sequence in undifferentiated (or differentiated) cells and (ii) the reviewer is unable to offhand suggest a known Brm target gene in differentiated or undifferentiated myoblasts.

Nevertheless, there is precedence for such a possibility. Moran's lab (JBC 284:10067, 2009) showed in an osteoblast differentiation system that both Brg1 and Brm were present on target promoters prior to gene activation and that Brm functionally acted as a repressor of Brg1 because Brm depletion accelerated differentiation and gene activation whereas Brg1 depletion prevented differentiation and gene activation. In the absence of any contradictory data, one could imagine that the MyoD-Baf60c complex present at the myogenin prior to differentiation also contains Brm and that this complex acts as a repressor that is displaced upon differentiation induced p38 activity that drives binding of a phosphorylated Baf60c complex that contains Brg1 and other SWI/SNF

subunits.

On a related note, on p. 8, the authors statement "Our results showing that the MyoD-BAF60c complex could first be recruited to the myogenin promoter in the absence of the ATPases Brg1 and Brm...." is not well supported with regard to Brm and perhaps should be modified.

Other, more minor concerns (in order of presentation):

- 1) In the text on page 6, the authors describe Supp. Fig. 2 but refer to Supp. Fig. 3. Supp. Fig. 2 is inadequately controlled because the authors do not show western blots to confirm the proteins targeted by siRNA were reduced. This is puzzling because other knockdown experiments in the manuscript have such controls.
- 2) The experiments in Supp. Fig. 4 do not directly address the conclusion that Baf60b is an "ancillary" factor because in each model system tested, the data only reinforce the importance of the Baf60c protein. A comparison of Baf60c and Baf60b experiment in these different model systems possibly would have more rigorously supported the authors' prediction about the role and relative importance of Baf60b.
- 3) Figure 3C shows residual phosphorylation of Baf60cThr229Ala in an SB independent manner. This should be acknowledged. In addition, the text on page 9 should be altered from "SB prevented the co-IP of Brg1 with Baf60c" to "SB reduced the efficiency of co-IP of Brg1 with Baf60c".
- 4) Figure 4A-B are improperly controlled. These experiments lack western blots to demonstrate the extent of Baf60c overexpression and flag westerns to demonstrate the expression of the ectopic Baf60c protein. Moreover, differentiation and target gene expression due to overexpression of wt or mutant Maf60c should be compared to an empty vector control; this should not be "data not shown".
- 5) There is a significant word choice error in the top section of page 10, where the authors indicate "Brg1 was associated ... in C2C12 myoblasts" when the data indicate a Brg1 association in myotubes or differentiated myoblasts.
- 6) Figure 5D -E
Fig. 5D has no y-axis label.
Fig. 5E refers to SWI/SNF "core". "Core" is vague and not informative. It is not a "core" complex simply because it is derived from HeLa cells. It should simply be called "HeLa SWI/SNF" or something similar.
There is no data presented about the integrity of the chromatin template assembled in vitro. Surely some characterization was performed. Is there something that can be shown? Were similar experiments done using naked DNA templates?
- 7) The data in Supp. Figure 6 on H3K9me3 should be reconsidered. The increase described by the authors when Baf60c is knocked down is less than 2-fold, and though it appears to be significantly different from the scrambled GM control, the relatively large error bars in the BAF60 knockdown under growth conditions makes the conclusion of an increase doubtful. This piece of data is not directly related to the rest of the manuscript. Is it necessary?
- 8) The image quality of Fig. 6A is very poor. The numbers on the x- and y-axes cannot be read.

1st Revision - authors' response

24 August 2011

Referee #1

1. The authors have very convincingly shown that BAF60c (but not Brg1) associates with the myogenin promoter in proliferating myoblasts. It would be nice if the authors could extend this analysis to include other subunits of the Swi/Snf complex-to address whether BAF60c is indeed the only member of this complex to associate (with MyoD and/or the myogenin promoter) in proliferating myoblasts (or in cells treated with SB203580).

RE: Indeed, we do show that one essential component of the SWI/SNF complex (Ini1 or BAF47) is present on myogenin promoter in DM (together with MyoD, BAF60c, Brg1), but not in GM (when only BAF60c and MyoD are detected). Because Ini1 is one typical structural SWI/SNF subunit, we believe that this evidence supports the conclusion that the “conventional” Brg1-based SWI/SNF complex does not associate with MyoD/BAF60c in proliferating myoblasts. We do agree with the reviewer that knowing the precise composition of the complexes in GM and DM is an important issue, but we also note that this will require additional extensive studies, including detailed proteomic analysis, that should be the object of a distinct project.

2. *The authors demonstrate that phosphorylation of BAF60c by p38alpha/beta is necessary to induce the interaction of BAF60c with Brg1 in vitro, and recruit Brg1 to the myogenin promoter in differentiated myocytes. However, along the same lines as point 1, it would be nice for the authors to evaluate whether the interaction between BAF60c and Brg1 is necessary to recruit other components of the Swi/Snf complex. One could do this by addressing whether knockdown of Brg1 reduces the association of other components of the Swi/Snf complex with the myogenin promoter in myocytes.*

RE: The reviewer suggests a very interesting experiment; however, again we argue that knowing the identity of the SWI/SNF component(s) that mediate interactions with phosphorylated BAF60c a of the complexes in GM and DM will require additional, extensive studies that are behind the scope of the current manuscript.

Referee #2

This reviewer states that “if this truly is a “take it or leave it” decision, the reviewer casts an unequivocal vote for acceptance”. However, he/she mentions one conceptual shortcoming “that.....could be addressed in the future by the authors or others if the authors don't already have experimental evidence in hand to present in this manuscript”.

The authors' model suggests a MyoD-Baf60c complex on the myogenin promoter prior to myogenic differentiation and gene activation that is devoid of the Brg1 ATPase found in many SWI/SNF complexes. However, the authors do not account for the possibility that the related SWI/SNF ATPase, Brm, could be present at the promoter prior to differentiation. Although the authors indicate that they found no evidence for a stable association of Brm with MyoD in their protein interaction studies, this is negative data that could be influenced by experimental conditions. A more definitive experiment would be a directed ChIP experiment, though (i) a negative result would only be meaningful if there were a positive control showing Brm binding to another sequence in undifferentiated (or differentiated) cells and (ii) the reviewer is unable to offhand suggest a known Brm target gene in differentiated or undifferentiated myoblasts.

Nevertheless, there is precedence for such a possibility. Moran's lab (JBC 284:10067, 2009) showed in an osteoblast differentiation system that both Brg1 and Brm were present on target promoters prior to gene activation and that Brm functionally acted as a repressor of Brg1 because Brm depletion accelerated differentiation and gene activation whereas Brg1 depletion prevented differentiation and gene activation. In the absence of any contradictory data, one could imagine that the MyoD-Baf60c complex present at the myogenin prior to differentiation also contains Brm and that this complex acts as a repressor that is displaced upon differentiation induced p38 activity that drives binding of a phosphorylated Baf60c complex that contains Brg1 and other SWI/SNF subunits.

RE: We agree that the reviewer is raising an interesting issue, which could be addressed in the future by the authors. Indeed, a research is currently undergoing in our lab to determine the individual roles of the SWI/SNF ATP-ase subunits, Brg1 and Brm in proliferating and differentiated skeletal muscle cells. Our data shows that Brm does not bind myogenin promoter in proliferating, undifferentiated myoblasts (GM); however, we detected Brm on myogenin promoter at late time points of differentiation (48 hours of DM culture). Consistently, genome-wide gene expression analysis performed in Brm-depleted cells revealed that Brm is required for the expression of muscle genes (including myogenin) at late time points. The reviewer will certainly understand that this data belongs to a different manuscript that is currently in preparation and it would be preferable not to publish any of these results in the present manuscript. Still, we believe that it was fair to share with the reviewers the information in the rebuttal letter. This way we also make available the information to the readers that will access this letter through the EMBO website.

On a related note, on p. 8, the authors statement "Our results showing that the MyoD-BAF60c complex could first be recruited to the myogenin promoter in the absence of the ATPases Brg1 and Brm...." is not well supported with regard to Brm and perhaps should be modified.

RE: We have modified the text accordingly

Other, more minor concerns (in order of presentation):

- 1) *In the text on page 6, the authors describe Supp. Fig. 2 but refer to Supp. Fig. 3. Supp. Fig. 2 is inadequately controlled because the authors do not show western blots to confirm the proteins targeted by siRNA were reduced. This is puzzling because other knockdown experiments in the manuscript have such controls.*

RE: The reviewer is correct regarding the mistake on referring to Supp. Fig. 3 instead of Supp. Fig. 2. As for the western blots of proteins targeted by siRNA, we could not show the protein levels of BAF60b because there is no specific antibody available. Thus, we decided to rely on RNA levels to monitor the efficiency of RNAi. The downregulation of BAF60c protein by RNAi is shown in Suppl Fig. 4, using the anti-BAF60c antibody generated by us.

- 2) *The experiments in Supp. Fig. 4 do not directly address the conclusion that Baf60b is an "ancillary" factor because in each model system tested, the data only reinforce the importance of the Baf60c protein. A comparison of Baf60c and Baf60b experiment in these different model systems possibly would have more rigorously supported the authors' prediction about the role and relative importance of Baf60b.*

RE: The direct comparison of gene affected by BAF60b and BAF60c RNAi can be made from data shown in Suppl. Figs 3 and 4 and from the list of genes down-regulated by siRNA-mediated depletion of each of these SWI/SNF sub-units - accessible through GEO Series accession number GSE24573 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24573>). As only a small subset of muscle genes downregulated by BAF60c RNAi is also downregulated by BAF60b RNAi we found appropriate to define BAF60b as an ‘ancillary’ factor, in the absence of further mechanistic insight on this apparent redundancy between BAF60c and b.

- 3) *Figure 3C shows residual phosphorylation of Baf60cThr229Ala in an SB independent manner. This should be acknowledged. In addition, the text on page 9 should be altered from "SB prevented the co-IP of Brg1 with Baf60c" to "SB reduced the efficiency of co-IP of Brg1 with Baf60c".*

RE: The residual phosphorylation seen in the BAF60c mutant manner is due to by the residual, non-specific binding of radioactive ATP, after washing, during the kinase assay. This activity is p38 independent, since was not eliminated by SB. On page 9, we replaced the sentence "SB prevented the co-IP of Brg1 with Baf60c" with "SB reduced the efficiency of co-IP of Brg1 with Baf60c".

- 4) *Figure 4A-B are improperly controlled. These experiments lack western blots to demonstrate the extent of Baf60c overexpression and flag westerns to demonstrate the expression of the ectopic Baf60c protein. Moreover, differentiation and target gene expression due to overexpression of wt or mutant Maf60c should be compared to an empty vector control; this should not be "data not shown".*

RE: We have introduced in the revised manuscript, both the western blot of ectopically expressed BAF60c proteins and the effect of empty vector control on phenotypic differentiation and target gene expression.

- 5) *There is a significant word choice error in the top section of page 10, where the authors indicate "Brg1 was associated ... in C2C12 myoblasts" when the data indicate a Brg1 association in myotubes or differentiated myoblasts.*

RE: The sentence referred to C2C12 confluent myoblasts – see also reply to reviewer one on Fig. 4 – and has been modified in the revised text to explain that this experiment was performed in confluent myoblasts.

- 6) *Figure 5D –E Fig. 5D has no y-axis label.*

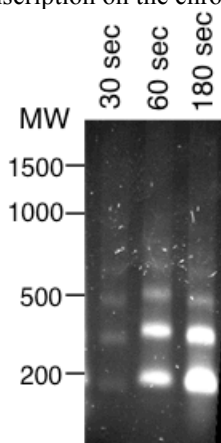
RE: We have included it in the revised manuscript.

Fig. 5E refers to SWI/SNF "core". "Core" is vague and not informative. It is not a "core" complex simply because it is derived from HeLa cells. It should simply be called "HeLa SWI/SNF" or something similar.

RE: We agree, and we have changed "core" complex with "HeLa SWI/SNF", as proposed by the reviewer..

There is no data presented about the integrity of the chromatin template assembled in vitro. Surely some characterization was performed. Is there something that can be shown? Were similar experiments done using naked DNA templates?

RE: We now provide, in the rebuttal letter, the MNase digestion of the chromatin that was used for the in vitro transcription studies (see figure below). This is the standard technique used to demonstrate that the plasmid has been incorporated into nucleosomal arrays. We did not perform the same transcription reaction on naked DNA templates. Activator-dependent transactivation can be very difficult to observe on naked DNA templates due to background transcriptional activity in the HeLa nuclear extracts. As such, we focused only on conditions for obtaining activator-dependent transcription on the chromatin templates.



MNase digestion of the chromatin that was used for the in vitro transcription studies

7) The data in Supp. Figure 6 on H3K9me3 should be reconsidered. The increase described by the authors when Baf60c is knocked down is less than 2-fold, and though it appears to be significantly different from the scrambled GM control, the relatively large error bars in the BAF60 knockdown under growth conditions makes the conclusion of an increase doubtful. This piece of data is not directly related to the rest of the manuscript. Is it necessary?

RE: We took the data out.

8) The image quality of Fig. 6A is very poor. The numbers on the x- and y-axes cannot be read.

RE: We have fixed the image.

Thank you for re-submitting your manuscript. As discussed, we returned the revision to one of the referees, whose comments are enclosed. The referee is broadly in favour of publication, pending satisfactory textual revision. Notably, the referee expressed disappointment that many of the issues reiterated in the second report (below) had not already been addressed in revision.

We agree with the referee that fig S14 cannot be considered a legitimate control experiment for the independent experiments presented in fig S12. We would still prefer addition of a formal expression

control, if this at all possible.

The actual controls requested in point 6 should indeed be added as SI information.

One key issue was the request for study of the MyoD-Baf60c complex prior to differentiation in light of Flowers et al., 2009. Your response was that this data is earmarked for another publication. The referee concurs with your plan. However, we agree with his/her request that the basic information has to be discussed prominently in the manuscript.

Also, please note a number of deficiencies with the data presented:

- 1) Please add scale bars throughout (figures 1,3,4,5, and SI 2,4 and 5).
- 2) There seems to be no statistical information provided (for example, figs. 2 and 6) - we require detailed description of the test applied the parameters (including description of what constitutes n). For low n, we request plotting the actual data points alongside the mean and error bars.
- 3) the contrast in panel 5d is too strong undermining the quantitative qualities of the data.
- 4) We now encourage authors to present key data in a second uncropped/unedited version to enhance the information content of the figure. We will add this information to each relevant figure as a 'source data' file.

I would like to invite you to submit a revised version of the manuscript, addressing the referee and the editorial comments, together with a point-by-point response to the referee report and the above issues.

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, please visit our website: <http://www.nature.com/emboj/about/process.html>

Please resubmit as soon as possible - ideally within 2-3 weeks - as there is limited time left for publication this year. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of 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.

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

Yours sincerely,

Editor
The EMBO Journal

REFeree REPORTS

Referee #2

I have reviewed the revised manuscript by Forcales et al. As I indicated in the first review, the manuscript should be accepted. However, some minor deficiencies still exist, and I recommend that these be considered (the conceptual issue) or addressed (the rest of the issues).

In my original review...

The conceptual issue was that although the data support the model, anyone familiar with the Flowers et al 2009 JBC paper from Moran's group could propose an alternative model that is consistent with the authors' results - namely that Brm is present on the myogenin promoter prior to differentiation as part of a repressive complex similar to what Flowers et al observed. I understand that the authors have a detailed study underway, and I completely agree that an in depth study of Brm function should not be required to publish the manuscript currently under consideration. However, I disagree that having the review process published online is an acceptable mechanism to make information available to readers. Personally, I (and most of my colleagues) have enough trouble keeping up with

the literature; the idea that most people would spend time reading the reviews and editorial correspondence about a paper as an additional source of new information seems unlikely to me.

A possible solution would be to discuss the Flowers et al result in a sentence in the discussion and add a statement that says that authors have no evidence at present for Brm binding to myogenin in myoblasts (data not shown), which suggests that a similar mechanism likely does not exist in this skeletal muscle differentiation model. The authors would be reporting a negative result, which would not need to be presented in data form. This strategy addresses the concern while maintaining the Brm-centered data set for future publication. However, I believe it should be left to the senior author to decide if this potential solution is acceptable.

Issue #1 indicated that the authors incorrectly referred to Supp. Fig. 3 on page 6 when they meant to refer to Supp. Fig. 2. In the first complete paragraph on page 6 of the original version, the authors made 4 references to Supp. Fig. 3. In the revised version, the text is located in the same place. Only two of the references to the Supp. Fig. have been corrected -lines 5 and 6 of that paragraph still refer to Supp. Fig. 3.

Issue #1 also indicated that the knockdown experiments in Supp. Fig. 2 was improperly controlled because the authors showed only mRNA levels and not protein levels. The authors responded that there is no Baf60b specific antibody, so the best that can be done is to present mRNA data. The reviewer acknowledges this point. However, the authors indicate that Baf60c westerns showing knockdown were presented in Supp. Fig. 4. The experiment in Supp. Fig. 2 is labeled as an siRNA experiment, and the methods section includes a protocol for transfecting siRNA molecules into C2C12 cells. The experiment in Supp. Fig. 4 is labeled as an shRNA experiment, and the methods section includes information on viral vectors encoding shRNA used to knock-down Baf60c.

It is not appropriate to refer to the Supp. Fig. 4 western as evidence that protein levels were reduced in Supp. Fig. 2 because the mechanism of knockdown was different in the two experiments.

In a perfect world, the authors would have performed a western on a duplicate sample when the experiments presented in Supp. Fig. 2 were performed. The absence of such a western in the original and in the revised version suggests that the authors do not have such data. At the revision stage, the authors might have just indicated that the requested data are not available and emphasized that the mRNA levels were reduced and there is a clear phenotype that is not observed when the scrambled siRNA control was used. I cannot see preventing publication of this story just because this western control was not performed, but neither can I withhold my objection to the suggestion that the Supp. Fig. 4 western should be accepted as evidence for knockdown in Supp. Fig. 2.

Issue #3 indicated that "Figure 3C shows residual phosphorylation of Baf60cThr229Ala in an SB independent manner. This should be acknowledged."

The authors responded "The residual phosphorylation seen in the BAF60c mutant manner is due to by the residual, non-specific binding of radioactive ATP, after washing, during the kinase assay. This activity is p38 independent, since was not eliminated by SB."

If the band represented nonspecific binding of radioactive ATP, then there should have been a band in lanes 1 and 4 of Fig. 3C, but there is not. Therefore the presence of the band is p38 dependent. I can accept that there might be residual binding of the radioactive ATP in the presence of p38. All that was requested was that this observation be acknowledged. A sentence in the figure legend or methods would have been adequate.

Issue #5 (also brought up by reviewer 1) questioned the text describing Fig. 4D (now Fig. 4E). The authors indicated that they clarified the text, but the new description (pasted below) is deficient. I'm reasonably sure that the authors meant to put the word "in" after "detectable" but I'd prefer that the authors review and modify the text themselves.

In confluent myoblasts (GMc) Brg1 recruitment to myogenin promoter was barely detectable C2C12 confluent myoblasts expressing empty vector; however, Brg1 chromatin was enhanced in C2C12 confluent myoblasts in which BAF60c wt was overexpressed, but not in BAF60c Thr229Ala-expressing cells (Figure 4E). This evidence further supports the conclusion that BAF60c phosphorylation by differentiation-activated p38 alpha/beta promotes the incorporation of MyoD-

associated BAF60c into a Brg1-containing complex.

Issue #6 asked about controls for chromatin assembly of the in vitro transcription template. The authors showed a micrococcal nuclease assay in their rebuttal. It would be preferable to either include this figure in the supplemental data or add a sentence to the methods to indicate that the control was performed and the digestion gave the expected nucleosome ladder.

Issue #7 questioned the data in Supp. Fig. 6 pertaining to H3K9me3. The authors removed the panel containing this data from the Supp. Fig. However, the text describing the data remains present in the revised manuscript on page 11, sentence starting on the 14th line under the subheading "Baf60c is required...". The text related to H3K9me3 should be removed.

Other text errors noticed:

Page 13, the last sentence of the 2nd paragraph references Kadam and Emerson, 2003. This should be Kadam and Emerson.

Page 13, the next to last line refers to de La Serna, 2006. This likely should be de La Serna, 2005.

Page 15, first sentence of the plasmids section - Auwerx is spelled incorrectly.

Page 16 in the Gene Expression section - Affymetrix is incorrectly spelled in the subheading and in the sentence 5 lines from the bottom of the page.

2nd Revision - authors' response

29 September 2011

Issue #1 indicated that the authors incorrectly referred to Supp. Fig. 3 on page 6 when they meant to refer to Supp. Fig 2. In the first complete paragraph on page 6 of the original version, the authors made 4 references to Supp. Fig. 3. In the revised version, the text is located in the same place. Only two of the references to the Supp. Fig. have been corrected -lines 5 and 6 of that paragraph still refer to Supp. Fig. 3.

RE: we have fixed it – we thank the reviewer for this.

Issue #3 indicated that "Figure 3C shows residual phosphorylation of Baf60cThr229Ala in an SB independent manner. This should be acknowledged." The authors responded "The residual phosphorylation seen in the BAF60c mutant manner is due to by the residual, non-specific binding of radioactive ATP, after washing, during the kinase assay. This activity is p38 independent, since was not eliminated by SB." If the band represented nonspecific binding of radioactive ATP, then there should have been a band in lanes 1 and 4 of Fig. 3C, but there is not. Therefore the presence of the band is p38 dependent. I can accept that there might be residual binding of the radioactive ATP in the presence of p38. All that was requested was that this observation be acknowledged. A sentence in the figure legend or methods would have been adequate.

RE: we have acknowledged this observation in the methods, as requested by the referee

Issue #5 (also brought up by reviewer 1) questioned the text describing Fig. 4D (now Fig. 4E). The authors indicated that they clarified the text, but the new description (pasted below) is deficient. I'm reasonably sure that the authors meant to put the word "in" after "detectable" but I'd prefer that the authors review and modify the text themselves. In confluent myoblasts (GMc) Brg1 recruitment to myogenin promoter was barely detectable C2C12 confluent myoblasts expressing empty vector; however, Brg1 chromatin was enhanced in C2C12 confluent myoblasts in which BAF60c wt was overexpressed, but not in BAF60c Thr229Ala-expressing cells (Figure 4E). This evidence further supports the conclusion that BAF60c phosphorylation by differentiation-activated p38 alpha/beta promotes the incorporation of MyoD-associated BAF60c into a Brg1-containing complex.

RE: we have corrected this sentence as indicated by the referee

Issue #6 asked about controls for chromatin assembly of the in vitro transcription template. The authors showed a micrococcal nuclease assay in their rebuttal. It would be preferable to either

include this figure in the supplemental data or add a sentence to the methods to indicate that the control was performed and the digestion gave the expected nucleosome ladder.

RE: we have introduced a sentence to the methods to indicate that the control was performed and the digestion gave the expected nucleosome ladder.

Issue #7 questioned the data in Supp. Fig. 6 pertaining to H3K9me3. The authors removed the panel containing this data from the Supp. Fig. However, the text describing the data remains present in the revised manuscript on page 11, sentence starting on the 14th line under the subheading "Baf60c is required...". The text related to H3K9me3 should be removed.

RE: the text related to H3K9me3 has been removed

Other text errors noticed:

Page 13, the last sentence of the 2nd paragraph references Kadam and Emerson, 2003. This should be Kadam and Emerson.

RE: fixed

Page 13, the next to last line refers to de La Serna, 2006. This likely should be de La Serna, 2005.

RE: fixed

Page 15, first sentence of the plasmids section - Auwerx is spelled incorrectly.

RE: fixed

Page 16 in the Gene Expression section - Affymetrix is incorrectly spelled in the subheading and in the sentence 5 lines from the bottom of the page.

RE: fixed

We also corrected the following deficiencies with the data presented, indicated by the editor:
1) Please add scale bars throughout (figures 1,3,4,5, and SI 2,4 and 5).

RE: scale bars have been introduced in all these figures

2) There seems to be no statistical information provided (for example, figs. 2 and 6) - we require detailed description of the test applied the parameters (including description of what constitutes n). For low n, we request plotting the actual data points alongside the mean and error bars.

RE: A statistical analysis for ChIP experiments has not been applied. The Chip experiments shown are representative of at least 2 independent experiments, as indicated in the materials and methods section. Most of the ChIP results published do not present a statistical analysis, the reason for this is that ChIPs from the same experimental conditions performed in different experimnts can have quite different numbers, although the trend has to be always the same (increases or decreases of enrichments). The graphics include the IgG, which is the background control. Anything above the IgG enrichments should be considered significant. In support of our claim, the following manuscripts that do not contain statistical analysis for ChIP experiments were randomly selected from latest issues of EMBO J.

Jensen et al. FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. The EMBO Journal (2011), 1–17 (Figure 5 and 6)

Pospisil et al. Epigenetic silencing of the oncogenic miR-17-92 cluster during PU.1-directed macrophage differentiation. The EMBO Journal 6 September 2011 (Figure 3-B-I)

Chikh et al. iASPP/p63 autoregulatory feedback loop is required for the homeostasis of stratified epithelia. The EMBO Journal 6 September

3) the contrast in panel 5d is too strong undermining the quantitative qualities of the data.

RE: Figure 5d is actually a graph. If the editor means another figure, please specify so we can work on it

4) We now encourage authors to present key data in a second uncropped/unedited version to enhance the information content of the figure. We will add this information to each relevant figure as a 'source data' file.

RE: we agree on this “transparency issue” and are willing to provide key rough data. However, in this specific circumstance the task is complicated by a number of adverse conditions. First, the

original data are quite scattered between the labs in Ottawa, San Diego and Rome where the experiments have been performed. By coincidence, we have just moved our lab in San Diego (where most of the data have been generated) from one building to another, and I have hundreds of unpacked boxes piled outside my office; on the other hand, Sonia Forcales, the first author of the manuscript, just moved to Barcellona where she is setting her own lab. She sent by mail the lab material (including most of the original data), but the packages have not arrived yet. I should also note that most of the western blots in our lab are typically cropped in origin – by cutting the membrane before blotting - to save precious antibodies. Because of these problems and given the time sensitive nature of this re-submission (editor requested to resubmit the manuscript as soon as possible - ideally within 2-3 weeks) we would like to ask the manuscript to be processed without this information, but are willing to provide it as soon as available.