β-actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming

Mohamed Al Sayegh, Raza Mahmood, Salema Abul Khair, Xin Xie, Mei El Gindi, Tina Kim, Alia Almansoori, and Piergiorgio Percipalle

Corresponding author(s): Piergiorgio Percipalle, New York University Abu Dhabi

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RE: E19-11-0628
TITLE: "Nuclear β-actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming"

Dear Dr. Percipalle,

Your manuscript, referenced above, has been read by two reviewers, whose comments are attached. The referees both agree that the work you describe is potentially of great interest to the field, addressing an important question in developmental control of cell differentiation. However, both felt that the mechanistic connection between nuclear beta-actin and the CEBPA gene was not strong enough to support your interpretations. The main issue raised by Referee 2 can be summarized in this sentence: "The case that it is nuclear actin that does this is not well made and it remains possible that the effects are indirect and arise from disruption of the cytoskeleton." Similarly, Referee 1 states: "This means that the basic expression of CEBPA does not depend on H3K9Me3 modification, Brg1 and CEBPB binding, or nuclear actin itself. What is the authors explanation for this? "

Based on these reviews, I am unable to proceed with publication of this work in MBoC. My experience tells me that this manuscript requires a great deal of new experimentation and bioinformatic analysis. Thus I am formally rejecting the paper.

I may consider a revised version provided you address each reviewer concern. However, I must tell you clearly that the reviewers have raised many issues that would require a substantial number of additional experiments; thus, you should carefully consider whether you are prepared to perform the large amount of work that would be necessary for me to consider a revised manuscript. If you do decide to go forward, please make sure to include a point-by-point response to each issue raised by the reviewers.

I hope that these comments will be useful to you as you prepare a revision of the manuscript, here at MBoC, or elsewhere.

Sincerely,

Greg Matera
Associate Editor
Molecular Biology of the Cell

Dear Dr. Percipalle,

The review of your manuscript, referenced above, is now complete. The reviewer comments are included below. The Monitoring Editor has decided that your manuscript is not acceptable for publication in Molecular Biology of the Cell. We hope that the Monitoring Editor's and reviewer comments will be helpful to you as you continue your work

Thank you for the opportunity to examine this work. We hope that as your studies progress you will consider submitting future manuscripts to Molecular Biology of the Cell.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript the authors describe their observation that nuclear actin is required for the maintenance of opened chromatin state during transcriptional reprogramming of pre-adipocytes. They show that during differentiation the regulatory activity of β-actin is performed through controlling chromatin accessibility at the region proximal from the Cebpa gene which is a key factor needed for adipocyte specific gene expression. The topic is interesting and the findings are novel. The manuscript meets the formal requirements. Nevertheless, there are some problems that require attention before I believe the paper will be ready for others to read.

1. The authors show ORO stainings (e.g. Fig 2B) multiple times. It would be nice if these stainings would be quantified.
2. Please indicate somewhere what the numbers mean (number of genes) in Fig1A and 2C.
3. On the heatmap in Figure 1C the wild type MEF-WT_4 sample looks exactly the same as the KO samples. What is the explanation for this deviation? Since there are 4 WT and 3 KO samples, I am afraid that the labels have been reversed. If this is the case, the conclusions drawn by the authors are false: Ebf2 is in fact strongly downregulated Ppargc1a, Rorc and Hmga2 are upregulated in the KO condition!
4. Figure 2D is not discussed in the text, and Fig 2E is mislabeled in the figure legend to C. They mention Socs1 in the text but Socs2 is written in Fig2E.
5. The immunoblots of CEBPA and FAB4 (p.7) in WT and KO adipocytes should be presented.
6. According to the result shown in Figure 3A, there is a low level expression of the Cebpa gene in uninduced wild type and actin KO MEF cells. The level of this expression is the same in the two cell types. In contrast to this, the H3K9Me3 pattern, Brg1 binding and ATAC-Seq results (Fig.4A), and moreover, the binding of CEBPB are significantly different in the non-induced WT and KO MEF cells (Fig.4B). This means that the basic expression of Cebpa does not depend on H3K9Me3 modification, Brg1 and CEBPB binding, or nuclear actin itself. What is the authors explanation for this?
7. Upon induction of differentiation, Cebpb mRNA level increases in wild type MEF cells. In actin KO cells the same increase in Cebpb mRNA production can be observed after induction (Figure 3A). Interestingly, after induction KO cells expressing NLS tagged actin exhibit a Cebpb mRNA level that is 2.5 times higher than in the WT or KO cells (compare Fig. 3A to Fig. 3E). This should be discussed since it nicely supports the model outlined by the authors.

Minor issues:
- The sentence „Expression of Cebpa gene is downregulated" in the Introduction should be merged with the previous sentence.
- Please specify how total variance was calculated in the Principal Component Analysis (Fig. 1SA).
Textual issues, typos, etc.:
- Introduction, 5th sentence: replace "WE" with "We".
- The citations for the Shapira papers are written in red, and the format of the second citation is incorrect.
- First sentence in the Results section: replace "adipocytes differentiation" with "adipocyte differentiation".
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- Figure legend for Fig.4. "See panel B for the location..." I think panel A would be correct here.

Reviewer #2 (Remarks to the Author):

Nuclear □-actin contributes to an open chromatin....
from Al-Sayegh and colleagues

While actin is now generally accepted that be present in cell nuclei, whether it plays any functional role in nuclear processes such as transcription has long been a matter of controversy. The Percipalle has previously used □-actin knockout cells to shown that □-actin suppressed methylation of H3K9 and H3K27, and was required in a chemical induced neuronal differentiation model, appears to play a role in recruiting the chromatin regulator Brg1 to chromatin, suggesting that actin controls chromatin changes during neural differentiation.

In this paper, they extend this study to look at the role of actin in the control of adipocyte differentiation.

First they show that □-actin knockout MEFs exhibit defects in expression of genes involved in far cell differentiation, showing both increases and decreases. Using an adipocyte reprogramming model, they show that □-actin knockout MEFs adipocytes exhibit enhanced adipocyte-like features under basal conditions, and a more pronounced increase in inducing conditions. This suggests that □-actin suppresses adiogenic differentiation.

INterestingly, however, in contrast to wildtype cells, induction of □-actin knockout MEFs shows no upregulation of Cbbpa, a pioneer TF implicated in adipocyte differentiation, or of STEAP4 and PSMB8, two other proteins required for adipocyte maturation. They also show that actin knockout cells lack an ATAC peak near the Cebpa gene, but do not look at what happens in this assay upon adipocyte induction..

The authors then look at whether actin directly affects Cebpa expression, and find that reepxression of an NLS-tagged □-actin (over)restores induction of the gene upon adipocyte differentiation (They do not show that the ATAC peak is restored, or prove that this effect requires nuclear-localized actin).

The authors propose that nuclear □-actin plays a role in maintaining open chromatin during

differentiation, and that this reflects an effect on recruitment of the BAF complex.

While the paper clearly shows that loss of □-actin impacts on adipocyte differention, and on adipocytic gene expression in undifferentiated MEFs, the study lacks any real mechanistic insight into the basis for this phenomenon. It is therefore not suitable for publication.

The case that it is nuclear actin that does this is not well made and it remains possible that the effects are indirect and arise from disruption of the cytoskeleton. Controls showing nuclear expression levels in the NA line should be shown, as well as experiments with a cytoplasmically restricted actin. They conclude that actin works on Cebpa expression by affecting recruitment of the BAF complex, but they present no data on this, and so the conclusions of the paper are entirely speculative.

It is also puzzling that the while authors see a dependence of Cebpa expression on actin, they don't see an effect of actin loss on adipocyte differentiation, which if anything appears to work better when actin is lost.

COMMENTS

Abstract - No data is presented on Brg1 in this paper, so the reference to it in the abstract should be removed.

Figure 2B, 3D - These figures must be quantified somehow - by numbers of lipid droplets / cells, amount of lipid, or oil-Red O staining intensity.

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The observation that □-actin knockout MEFs show no upregulation of Cdbpa in inducing conditions is a puzzle when it appears that these cells are more adipogenic than wildtype. How do the authors reconcile this observation with the notion that Cepba drives adipogenic differentiation through the PPARg pathway?

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Figure 3E - What does NLS actin expression in KO cells do to differentiation? one would assume that it suppresses it - is this the case? What happens in wildtype cells?
Figure 3E - what does NLS-actin expression do to expression of the STEAP4 and PSMB8 genes. Does it affect them in the same way?

Figure 4. In the ATAC experiments, what happens to the ATAC signal at Cebpa upon induction of adipogenesis? One would expect nothing, if the ATAC signal is related to gene induction - is that the case? What does NLS actin do to the ATAC signal at Ccebpa? Is this seen with cytoplasmically-restricted actin?

There is no presentation of the totality of the ATACseq data - how many other genes show ATAC peaks that disappear in the KO cells? What do these genes have in common? Is Brg1 recruited to them?

p8 bottom - the CEBPB site is presumably 800bp from the TSS - but it looks like 300bp instead! Fix this.

Figure S2 PSMB8 is much more severely affected by the KO than Cebpa, yet there is no effect on the ATAC signal. How do the authors reconcile this with their model that actin is controlling the induction of Cebpa / PSMB8 / STEAP4 upon adipogenesis?

p9 The authors conclude that actin directly regulates accessibility of the cebpa promoter to CEBPB, but provide no evidence of this.

p9 There is no heading for the Discussion section.

POINT-BY-POINT RESPONSES TO THE REVIEWERS' COMMENTS

We would like to thank all reviewers for their comments that overall have given us the possibility to improve the manuscript.

Responses to reviewer #1

In this manuscript the authors describe their observation that nuclear actin is required for the maintenance of opened chromatin state during transcriptional reprogramming of pre-adipocytes. They show that during differentiation the regulatory activity of β-actin is performed through controlling chromatin accessibility at the region proximal from the Cebpa gene which is a key factor needed for adipocyte specific gene expression. The topic is interesting and the findings are novel. The manuscript meets the formal requirements. Nevertheless, there are some problems that require attention before I believe the paper will be ready for others to read.

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We have included quantification of ORO staining on MEFs and induced adipocytes (MEF-iA) in wt and KO conditions (see new bar diagram in figure 2C) and in KO cells where beta actin has been reintroduced both in the MEF and induced adipocyte condition (see figure 3, panel E)

2. Please indicate somewhere what the numbers mean (number of genes) in Fig1A and 2C.

We have specified that the numbers refer to gene numbers (see legends to figure 1A and 2C)

3. On the heatmap in Figure 1C the wild type MEF-WT_4 sample looks exactly the same as the KO samples. What is the explanation for this deviation? Since there are 4 WT and 3 KO samples, I am afraid that the labels have been reversed. If this is the case, the conclusions drawn by the authors are false: Ebf2 is in fact strongly downregulated Ppargc1a, Rorc and Hmga2 are upregulated in the KO condition!

Thanks for noticing this. Indeed, there was some confusion with the labels. As can be seen we have generated new heat maps (without MEF-WT_4) and the results support downregulation of Ppargc1a, Rorc and Hmga2 in the KO condition and Ebf2 is upregulated

4. Figure 2D is not discussed in the text, and Fig 2E is mislabeled in the figure legend to C. They mention Socs1 in the text but Socs2 is written in Fig2E.

Figure 2D (now figure 2E) is now discussed in the revised text. We have also fixed the mislabeling in the corresponding figure legend, see page 6.

5. The immunoblots of CEBPA and FAB4 (p.7) in WT and KO adipocytes should be presented.

As requested, we have now included immunoblots for CEBPA, CEBPB and FABP4 (see figure 3 in the revised manuscript).

6. According to the result shown in Figure 3A, there is a low level expression of the Cebpa gene in uninduced wild type and actin KO MEF cells. The level of this expression is the same in the

two cell types. In contrast to this, the H3K9Me3 pattern, Brg1 binding and ATAC-Seq results (Fig.4A), and moreover, the binding of CEBPB are significantly different in the non-induced WT and KO MEF cells (Fig.4B). This means that the basic expression of Cebpa does not depend on H3K9Me3 modification, Brg1 and CEBPB binding, or nuclear actin itself. What is the authors explanation for this?

Expression of CEBPA is considerably downregulated in the beta actin KO cells (see Figure 3A) both in the MEFs and in the induced adipocytes. We believe that this is due to increased heterochromatin levels at the regulatory region upstream the transcription start site (TSS) of the CEBPA gene. In fact, figure 4 panel A shows that immediately upstream the TSS of the CEBPA gene there is increased H3K9Me3, loss of Brg1 and loss of ATAC signal in the absence of beta actin. Increased heterochromatin and, therefore, a more compact chromatin phenotype, is likely to be a consequence of impaired Brg1 deposition (see Xie et al., 2018, FASEB J) and increased H3K9Me3 (as previously shown in Xie et al., 2018, FASEB J and Xie et al., 2018 PLOS Genetics).

In the revised version of the manuscript we show evidence that this chromatin compaction affects CEBPB binding. In support of this conclusion, we have generated new ChIP qPCR data (see figure 4, B-D and supplementary Figure S2, Panels F-I) on chromatin isolated from wt and KO before and after reprograming to adipocytes as well as on chromatin isolated from MEF expressing NLS tagged beta actin in the nucleus of KO cells before and after reprogramming. To find out whether CEBPB binding is affected possibly due to concomitant changes in chromatin compaction, we designed primers targeting specific loci in the upstream regulatory region and some controls (including loci within the gene and further away from the regulatory region). Remarkably, results from the new analysis added as part of figure 4 show that in wt cells CEBPB binds normally to region 5 both in the MEF and induced adipocyte condition and we do not see any increase in H3K9Me3 levels. However, in the same region but in the KO cells binding of CEBP is lost both in the MEF and induced adipocyte condition whereas there are increased levels of H3K9Me3. This suggests that loss of beta actin leads to loss of CEBPB binding due to changes in heterochromatin levels/chromatin compaction during reprograming. This effect is due to nuclear actin. In fact in the same region (R5) CEBPB binding is rescued in beta actin KO cells expressing NLS-tagged beta actin in the cell nucleus both in the MEF and in the induced adipocyte conditions. Remarkably, in the same region we do not see any increase in H3K9Me3 levels. This suggests that actin dependent regulation of heterochromatin is required for CEBPB binding and, thus, activation of the CEBPA gene. In the absence of beta actin, given the ATAC-seq, it is likely that increased H3K9me3 levels are a consequence of impaired Brg1 deposition.

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Thanks for this, in the revised version of the manuscript we have introduced a few words to stress this specific point.

Minor issues:
- The sentence „Expression of Cebpa gene is downregulated" in the Introduction should be merged with the previous sentence.

This is now fixed in the revised manuscript

- Please specify how total variance was calculated in the Principal Component Analysis (Fig. 1SA).

This is now included in the figure legend to Figure S1

Textual issues, typos, etc.:
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- Figure legend for Fig.4. "See panel B for the location..." I think panel A would be correct here.

We have gone through the above typos and fixed where appropriate in the revised version of the manuscript.

Response to reviewer #2

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from Al-Sayegh and colleagues

While actin is now generally accepted that be present in cell nuclei, whether it plays any functional role in nuclear processes such as transcription has long been a matter of controversy. The Percipalle has previously used β-actin knockout cells to shown that β-actin suppressed methylation of H3K9 and H3K27, and was required in a chemical induced neuronal differentiation model, appears to play a role in recruiting the chromatin regulator Brg1 to chromatin, suggesting that actin controls chromatin changes during neural differentiation.

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It is also puzzling that the while authors see a dependence of Cebpa expression on actin, they don't see an effect of actin loss on adipocyte differentiation, which if anything appears to work better when actin is lost.

We have now extended the original study to include mechanistic insights supporting the role of nuclear actin in regulating heterochromatin at specific loci upstream the TSS of the CEBPA gene. Expression of CEBPA is considerably downregulated in the beta actin KO cells (see Figure 3A) both in the MEFs and in the induced adipocytes. We believe that this is due to increased heterochromatin levels at the regulatory region upstream the transcription start site (TSS) of the CEBPA gene. In fact, figure 4 panel A shows that immediately upstream the TSS of the CEBPA gene there is increased H3K9Me3, loss of Brg1 and loss of ATAC signal in the absence of beta actin. This per se suggests that actin-dependent deposition of Brg1 is impaired (see Xie et al., 2018, FASEB J) as a consequence of increased H3K9Me3 (as previously shown in Xie et al., 2018, FASEB J and Xie et al., 2018 PLOS Genetics) and more compact chromatin.

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We believe these new results included in the revised version of the manuscript make a case on the role of nuclear actin rather than the cytoplasmic pool of actin.

We agree with this reviewer's comments that it is puzzling to see increased adipogenic features in the absence of beta actin. After quantifying ORO staining we found that overall there is a lower degree of ORO staining in the absence of beta actin after adipogenic induction (see quantifications).

COMMENTS

Abstract - No data is presented on Brg1 in this paper, so the reference to it in the abstract should be removed.

We do not agree with this statement, we have ChIP-seq data showing that Brg1 is lost

from the regulatory region immediately upstream the TSS of the CEBPA gene in an actin-dependent manner (see Figure 4A)

Figure 2B, 3D - These figures must be quantified somehow - by numbers of lipid droplets / cells, amount of lipid, or oil-Red O staining intensity.

We have included quantification of ORO staining intensity on MEFs and induced adipocytes (MEF-iA) in wt and KO conditions (see new bar diagram in figure 2C). We have also performed quantification of the ORO staining on KO cells where beta actin has been reintroduced in both MEFs and induced adipocyte condition (see figure 3, panel E)

Figure 2C /p6 The authors should comment about the other gene sets affected in the knockout - chrondrocyte differentiation, osteoblast differentiation, etc.

We agree with this reviewer that a comprehensive analysis of all genes involved in chondrocyte differentiation and osteoblast differentiation is important. However, since this study focuses on adipogenesis, we believe that such an in-depth analysis on genes unrelated to adipogenesis goes beyond the scope and, in fact, it is part of another study which is currently ongoing.

FIgure 2D is not cited anywhere in the text

Figure 2D, currently figure 2E in the revised version of the manuscript, is now mentioned in the text.

"Principal-component" is spelt wrongly throughout

This is now fixed.

The observation that β-actin knockout MEFs show no upregulation of Cdbpa in inducing conditions is a puzzle when it appears that these cells are more adipogenic than wildtype. How do the authors reconcile this observation with the notion that Cepba drives adipogenic differentiation through the PPARg pathway?

Quantifications of the ORO staining before and after adipogenesis induction suggest that overall there is less lipid droplet production in the KO cells compared to wild type at the end of the differentiation process, an observation that is compatible with dysregulation of adipogenesis in vitro. Our results show that dysregulation is nuclear actin-dependent. Loss of beta actin induces chromatin compaction and this leads to inactivation of the CEBPA gene. This chromatin and transcription phenotype can be rescued when reintroducing nuclear actin. Therefore, it is unclear why our results are puzzling for this reviewer.

FIg 3 / p7 How come FABP4 expression is not seen in the RNAseq data presented in Figures 1 and 2?

As can be seen in the qPCR analysis and immunoblots, FABP4 expression is not significantly changed. This means that FABP4 is not differentially expressed between the wt and ko condition and therefore it is not expected to be present in the heatmap in figures 1 and 2. For this reason, it is also a good control to support specificity of the

effect that nuclear actin has on CEBPA gene activity.

FIgure 3A - the presentation here is strange. These are qPCR normalized to a housekeeping gene, and then plotted with uninduced MEF and KO cells set to 1. This is rather misleading, as the relative basal expression level in KO cells relative to WT is lost. The data should be replotted.

The data have been replotted accordingly (see figure 3 in the revised manuscript)

Figure 3E - this shows that reexpression of an NLS-tagged actin can restore induction of CEBPa to knockout cells - but the reexpression induces 100x increase rather than 3x. This Why is this? What expression level is the NLS actin compared with wildtype cells, have the authors ensured that it is comparable? Does untagged actin work in this experiment? What happens with cytoplasmically restricted actin? what happens if actin or NLS actin are overexprssed in wildtype cells?

KO MEFs expressing NLS-tagged beta-actin have been extensively characterized in previous studies (see Xie et al FASEB J, 2018 and Xie et al., iScience 2018). The expression levels of actin reintroduced into the cell nucleus are comparable to the amount of endogenous nuclear actin in wt condition.

As per the above questions raised by this reviewer, we do not have a mechanistic explanation but the fact that there is such a high, reproducible increase in CEBPA mRNA production upon reintroduction of beta actin in the cell nucleus speaks in favor of a direct role of nuclear beta actin in CEBPA gene activation and should not be a source of concern. Expression of untagged beta actin or expression of beta actin forced in the cytoplasm are both good ideas but may not be feasible in this specific context. If we constitutively express untagged beta actin, the exogenous protein behaves just like the endogenous wt protein. A considerable fraction of the reintroduced actin is imported in the nucleus and therefore there is a rescue of the phenotype. The second suggestion is trickier. It is not sustainable to constitutively express beta actin in the cytoplasm in the KO MEF background. Since there is a general dysregulation of transcription and chromatin, cells are not viable and loss of nuclear actin affects transcription and many nuclear processes and it is not sustainable for the cell over time. The option of blocking nucleocytoplasmic transport with specific drugs or by transient RNAi-mediated silencing of actin import/export receptors is valid but lacks specificity and the experiments would not be comparable with the experiments we have already performed.

Figure 3E - What does NLS actin expression in KO cells do to differentiation? one would assume that it suppresses it - is this the case? What happens in wildtype cells?

NLS actin reintroduced in the KO background does not suppress differentiation. Rather, we think it rescues the phenotype from the KO condition since it is able to rescue CEBPA expression (see Figures 3 and 4).

Figure 3E - what does NLS-actin expression do to expression of the STEAP4 and PSMB8 genes. Does it affect them in the same way?

As mentioned above, we have expressed an NLS-tagged version of beta actin in the nucleus of KO MEFs and measured the amounts of CEBPA mRNA before and after reprograming to adipocytes. As expected, the results from qPCR analyses shown in the

new figure 3, panel F, indicate that reintroduction of beta actin in the nucleus of KO cells leads to a rescue in the levels of CEBPA mRNA. This rescue is observed both in MEFs and in the corresponding induced adipocytes in the cells where nuclear actin was reintroduced.

Although it would be interesting to know what happens to Steap4 and PSMB8 once NLS actin is reintroduced in the KO background, understanding the precise mechanisms remains difficult at this stage and goes beyond the scope of this investigation. In fact, while Steap4 and PSMB8 RNA levels change in the absence of beta actin (see heatmap in figure 2) the chromatin across their genes is not altered (see supplementary figure S2). This suggests that the regulatory mechanisms are different from the ones that control CEBPA expression and might not be dependent on actin. In fact, we believe that changes in Steap4 and PSMB8 RNA levels are due to the loss of function of upstream factors such as CEBPA. We are definitely interested in finding out the details and we are currently doing this as part of a follow up investigation.

Figure 4. In the ATAC experiments, what happens to the ATAC signal at Cebpa upon induction of adipogenesis? One would expect nothing, if the ATAC signal is related to gene induction - is that the case? What does NLS actin do to the ATAC signal at Ccebpa? Is this seen with cytoplasmically-restricted actin?

These are very interesting questions. We do not have ATACseq data in differentiated cells but we plan to do these experiments as part of follow up projects.

To address the above questions, we have performed ChIP experiments with antibodies against CEBPB and H3K9Me3 (an epigenetic mark for repressive chromatin) in wt and KO cells before and after reprograming to adipocytes. Occupancies were studied at multiple regions immediately upstream the transcription start site (see R1-R5), inside the gene and further upstream. As can be seen in figure 4 panels B-D, in the specific region R5 CEBPB occupancy is specifically lost in the KO cells and, concomitantly with loss of CEBPB binding, we have increased H3K9Me3 in KO MEFs and corresponding induced adipocytes. Together with the ChIPseq analyses and ATACseq analyses shown in panel A, these new results indicate that loss of CEBPB binding at region R5 but not in the other regions (see figure 4B-D and supplementary figure S2, panels F-I) is compatible with increased heterochromatin formation/chromatin compaction at specific loci in the absence of beta actin, presumably by regulating Brg1 deposition and chromatin compaction (see Xie et al 2018, FASEB J). In support for a direct role of beta actin in this context, the same analyses conducted on MEFs and corresponding induced adipocytes expressing actin in the cell nucleus displays a rescue of CEBPB binding and loss of H3K9Me3 in the same region R5. In support of the specificity of CEBPB binding at region R5, we did not detect significant binding in the other regions analyzed. This is particularly interesting because it seems that R5 contains a putative CEBPB binding site.

Altogether, we believe these results address the above questions raised by this reviewer and provide initial mechanistic insights into nuclear actin-dependent regulation of heterochromatin during adipocyte differentiation.

There is no presentation of the totality of the ATACseq data - how many other genes show ATAC peaks that disappear in the KO cells? What do these genes have in common? Is Brg1 recruited to them?

Global analysis of the ATACseq data is part of a study that includes HiC seq data and is currently submitted for publication. It is also submitted in the bioRXiv preprint repository. Indeed, recruitment of Brg1 correlates with genes that have alterations in chromatin compaction.

p8 bottom - the CEBPB site is presumably 800bp from the TSS - but it looks like 300bp instead! Fix this.

This has now been fixed in figure 4

Figure S2 PSMB8 is much more severely affected by the KO than Cebpa, yet there is no effect on the ATAC signal. How do the authors reconcile this with their model that actin is controlling the induction of Cebpa / PSMB8 / STEAP4 upon adipogenesis?

We agree that PSMB8 is affected. However, we have not and do not claim anywhere in the manuscript that actin controls induction not only of Cebpa but also PSMB8 and STEAP4. Both PSMB8 and STEAP4 are downstream to Cebpa and therefore, it is likely that the effect of nuclear actin is only indirect on these two genes. In support of this conclusion although their mRNA levels are significantly altered the chromatin across their genes is not affected (see supplemental data), indicating that their regulation is likely to happen through a different mechanism.

p9 The authors conclude that actin directly regulates accessibility of the cebpa promoter to CEBPB, but provide no evidence of this.

As discussed above in detail, we do provide new evidence that actin is indeed required to facilitate access to CEBPB for binding to the CEBPA regulatory region by controlling a chromatin landscape that is compatible with transcription (as mentioned above). Also, please see the new figure 4 panels B-D and supplementary figure 2S panels F-I.

p9 There is no heading for the Discussion section.

A discussion section is not required in the short article format of the journal. Please see instructions for authors provided by the Journal.

RE: Manuscript #E19-11-0628R-A
TITLE: Nuclear β-actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming

Dear Dr. Percipalle:

Thanks for your patience as your manuscript advanced through the peer-review process. It was first seen by the original two reviewers, one of whom recommended to accept and the other to reject. I therefore sent the manuscript to a third reviewer to get an independent evaluation. As you will see, Reviewer #3 shares some concerns expressed previously by Reviewer #2 regarding the lack of convincing evidence for a specific role of nuclear actin. They suggest an additional experiment to express cytosolically-restricted beta-actin (NES-tagged) to rule out any role of cytoplasmic actin on CEPBA regulation. They alternatively suggest that you revise your conclusions and title to reflect the fact that you cannot conclusively implicate the pool of actin in the nucleus, although I suspect this option is not one that you would favor. Reviewer #3 also has a few questions relating to adipogenic differentiation that need to be answered.

I do appreciate that this represents yet another hurdle to overcome. If you decide to submit a revised manuscript that addresses these concerns, I will endeavor to evaluate it without further peer review, although I will reserve the option to send it back to the third reviewer depending on your responses.

Sincerely,

Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Percipalle,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have made a conscientious effort to address my previous criticisms. I have only one remark: please include a short description of the quantification of ORO staining also in the materials and methods section. (e.g. How did you measure OD514 on a microscope slide?)

Reviewer #3 (Remarks to the Author):

In the present manuscript entitled „Nuclear β -actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming" the authors used various NGS techniques in a β-actin KO MEF cell line to analyse β-actin dependent gene expression during transcriptional reprogramming. They describe their observation whereby β-actin depletion led to reduced Cebpa expression, a key factor for adipocyte differentiation, accompanied by a decrease in chromatin accessibility.
While the possible role of β-actin in the nucleus is gaining more attention and new findings regarding its function are being made, this manuscript still has some issues that need to be addressed.
How do the authors explain the presence of ORO positive lipid droplets in undifferentiated MF KO cells? Cells still differentiate into adipocytes in the absence of b-actin (Fig 2B+C). The authors claim that there are less ORO-positive cells in differentiated KO cells than compared to WT cells. How

was the quantification analysed? How do the MEF-iA KO cells compare to MEF WT and MEF-iA WT? In Figure 2C it seems like ORO O.D. of MEF-iA was quantified relative to its MEF control, therefore the relative increase after induction is lower in KO cells since their uninduced counterpart already shows lipid droplet staining.
The authors show that loss of b-actin leads to a loss of CEBPB binding to the regulatory region of Cebpa due to chromatin compaction and thus inactivation of CEBPA. However, why do the KO cells still undergo adipogenic differentiation even though the adipogenic pioneer factor CEBPA is inactivated?
Also, when comparing images and quantification of both MEF KO and MEF-NA cells (Fig. 2B+C, Fig. 3 D+E) they seem to behave similar after adipogenic induction.

The authors should include experiments after re-expression of cytosolically restricted b-actin (NES-tagged) to rule out any role of cytoplasmic actin on CEPBA regulation. This is important since actin (and many actin regulatory factors in the cytoplasm) is an essential cellular factor for MRTF-SRF dependent gene expression as several studies from the Treisman group and many others have shown. Otherwise, how do they really know that any of the observed effects are truly related to actin in the nucleus? Or else they should rephrase title and conclusions accordingly.

Minor points:
The authors repeatedly write that the CEBPB binding motif is located 800 kb upstream of the TSS (e.g. page 9). I should read "800 bp".
In the legend to Figure 4F the authors write "conditions were normalized to MEF KO". In the middle panel (Cebpb), however, fold induction of MEF KO was not set to "1" as has been done for the other two genes. Please correct or explain.
In the methods part to the ChIP-Seq experiment (p16) the authors mixed up the "distant upstream sequence (R6) as well as a sequence within the gene coding region (R7)" which should be the other way around according to figure 4A. Also, please add the missing catalogue number. Additionally, the authors write that they analysed regions (R1-R5) covering appr. 2 kb upstream of the Cebpa TSS. However, region R7, which is supposedly a "control region further upstream", still lies within the first 2000 bp upstream according to Figure 4A. How is the regulatory region upstream of the TSS defined?

Point-by-point responses to reviewers' comments, manuscript #E19-11-0628R-A

We would like to thanks the reviewers for the precious comments that have helped us improve the manuscript

Please find below our responses (in bold) to individual comments

1) Reviewer #1 (Remarks to the Author):

The authors have made a conscientious effort to address my previous criticisms. I have only one remark: please include a short description of the quantification of ORO staining also in the materials and methods section. (e.g. How did you measure OD514 on a microscope slide?)

Thanks for the positive feedback. We have now included a short description of the ORO staining quantification in the methods section

2) Reviewer #3 (Remarks to the Author):

In the present manuscript entitled „Nuclear β -actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming" the authors used various NGS techniques in a β-actin KO MEF cell line to analyse β-actin dependent gene expression during transcriptional reprogramming. They describe their observation whereby β-actin depletion led to reduced Cebpa expression, a key factor for adipocyte differentiation, accompanied by a decrease in chromatin accessibility.
While the possible role of β-actin in the nucleus is gaining more attention and new findings regarding its function are being made, this manuscript still has some issues that need to be addressed.

How do the authors explain the presence of ORO positive lipid droplets in undifferentiated MF KO cells?

Thanks for this important question. Although we do not have mechanistic insights, we and others have already reported that in the absence of beta actin cells are transcriptionally reprogrammed and transcripts known to be involved in adipogenesis, are upregulated. It is therefore not surprising that in the absence of beta actin cells display the presence of lipid droplets even prior to adipogenic reprograming.

Cells still differentiate into adipocytes in the absence of b-actin (Fig 2B+C). The authors claim that there are less ORO-positive cells in differentiated KO cells than compared to WT cells. How was the quantification analysed?

We have included a description of the quantification in the methods section. For analysis in figure 2C wt and KO MEFs were set to 1. Corresponding induced adipocytes were respectively compared with their respective MEFs.

How do the MEF-iA KO cells compare to MEF WT and MEF-iA WT? In Figure 2C it seems like ORO O.D. of MEF-iA was quantified relative to its MEF control, therefore the relative increase after induction is lower in KO cells since their uninduced counterpart already shows lipid droplet staining.

As mentioned above, induced adipocytes were compared with their corresponding initial MEF state. In other words, wt induced adipocytes were compared with wt MEFs and KO induced adipocytes were compared with KO MEFs. In our opinion it does not make sense to compare KO induce adipocytes with wt MEFs.

The authors show that loss of b-actin leads to a loss of CEBPB binding to the regulatory region of Cebpa due to chromatin compaction and thus inactivation of CEBPA. However, why do the KO cells still undergo adipogenic differentiation even though the adipogenic pioneer factor CEBPA is inactivated?

This reflects the fact that these cells are already transcriptionally reprogrammed. Although there is chromatin compaction at sites of CEBPB binding which interferes with full CEBPA gene activation during adipogenesis, there is a basal level of CEBPA transcription that is likely to be enough to maintain a minimal adipogenic state upon induction of adipogenesis.

Also, when comparing images and quantification of both MEF KO and MEF-NA cells (Fig. 2B+C, Fig. 3 D+E) they seem to behave similar after adipogenic induction.

These are different experiments and cell types and we can only speculate at this stage. ORO staining is a general marker for triglycerides and does not differentiate between different types of triglycerides. Given that we have major chromatin and transcriptional changes once actin is reintroduced in the cell nucleus, we believe that MEF-KO and MEF-NA cells only apparently display similar behavior in terms of lipid droplets production. They seem to produce lipid droplets that are different in morphology, nature and composition of triglycerides. Although this is an exciting observation we believe that it is more suitable for a follow-up project

The authors should include experiments after re-expression of cytosolically restricted b-actin (NES-tagged) to rule out any role of cytoplasmic actin on CEPBA regulation. This is important since actin (and many actin regulatory factors in the cytoplasm) is an essential cellular factor for MRTF-SRF dependent gene expression as several studies from the Treisman group and many others have shown. Otherwise, how do they really know that any of the observed effects are truly related to actin in the nucleus? Or else they should rephrase title and conclusions accordingly.

While we cannot completely discount the contribution of cytoplasmic b-actin, our observations suggest that the nuclear b-actin pool is the main factor regulating the phenotypes observed during adipogenic differentiation. We have shown that b-actin KO

cells exhibit dramatic changes in chromatin organization caused by the dysregulation of the b-actin containing BAF chromatin remodeling complex (Mahmood et al 2020 biorvx, Xie et al 2018). Since disruption of BAF/BRG1-mediated chromatin remodeling is directly related to the presence of b-actin inside the nucleus, we believe the chromatin accessibility changes observed in b-actin KO cells are primarily triggered by the dysregulation of the nuclear b-actin pool. While the Treisman lab, Vartiainen lab and others have demonstrated that changes in cytoplasmic or nuclear beta actin dynamics can regulate nuclear import of MRTF and activation of SRF-dependent pathways, such changes are primarily regulated at the level of transcription. Consistent with this idea, while our RNAseq analyses show that beta actin KO cells exhibit changes in both MRTF and SRF mRNA levels, neither the SRF nor the MRTF genes exhibit any significant changes in chromatin accessibility. In contrast, changes in CEBPA expression are accompanied by a noticeable change in chromatin accessibly pointing towards a chromatin-based mechanism distinct from the MRTF/SRF based transcriptional regulation. Together, these observations suggest that while both nuclear and cytoplasmic b-actin pools may influence regulatory pathways such as the MRTF/SRF, chromatin-based regulatory mechanisms are regulated primarily by the nuclear b-actin pool through its role in mediating BAF-dependent chromatin remodeling. Furthermore, while we have previously demonstrated that the expression of b-actin restricted to the cytoplasm does not reverse the phenotypes observed in b-actin KO cells (Xie et al 2018), as suggested by this reviewer, we have nevertheless rephrased the title and some of our concluding remarks to ensure that the possible role of cytoplasmic actin is not overlooked.

Minor points:
The authors repeatedly write that the CEBPB binding motif is located 800 kb upstream of the TSS (e.g. page 9). I should read "800 bp".

This is now fixed

In the legend to Figure 4F the authors write "conditions were normalized to MEF KO". In the middle panel (Cebpb), however, fold induction of MEF KO was not set to "1" as has been done for the other two genes. Please correct or explain.

Please note that figure 4F is the speculative model that we suggest. The figure legend reflects the figure.

In the methods part to the ChIP-Seq experiment (p16) the authors mixed up the "distant upstream sequence (R6) as well as a sequence within the gene coding region (R7)" which should be the other way around according to figure 4A.

Fixed

Also, please add the missing catalogue number. Additionally, the authors write that they analysed regions (R1-R5) covering appr. 2 kb upstream of the Cebpa TSS. However, region R7,

which is supposedly a "control region further upstream", still lies within the first 2000 bp upstream according to Figure 4A. How is the regulatory region upstream of the TSS defined?

The Cebpa gene regulatory region is broadly defined as the region containing transcription start site, promoter and putative promoter-proximal CEBPB binding sites

RE: Manuscript #E19-11-0628RR
TITLE: "β-actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming"

Dear Dr. Percipalle:

Thanks for submitting your revised manuscript to MBoC. I sent the manuscript back to two of the reviewers for their input, and as you can see both are now supportive of publication. However, one reviewer suggests you make a correction to the figure legends, which should be easy to incorporate. I also ask that you go carefully through the MBoC Author Submission Checklist and make sure that all of the relevant points are fully addressed. Once you return a suitably-revised manuscript I will be happy to accept it for publication.

Sincerely,
Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Percipalle,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In my opinion, the manuscript is suitable for publication in its present form.

Reviewer #3 (Remarks to the Author):

The authors have rephrased title and other lines as suggested. the sufficiently answered my concerns.
Minor:
I wrote previously : "In the legend to Figure 4F the authors write "conditions were normalized to MEF KO". In the middle panel (Cebpb), however, fold induction of MEF KO was
not set to "1" as has been done for the other two genes. Please correct or
explain.
I made a mistake here, and was actually referring to Figure 3F (not 4F). Maybe the authors still want to correct that.

Point-by-point responses to reviewers' comments, manuscript #E19-11-0628R-A

We would like to thanks the reviewers for the positive comments. Please find below our responses (in bold) to individual comments

1) Reviewer 1

In my opinion, the manuscript is suitable for publication in its present form.

Thanks for the positive feedback

2) Reviewer 3

The authors have rephrased title and other lines as suggested. the sufficiently answered my concerns.

Thanks for the feedback that has helped us improving the manuscript

Minor:

I wrote previously: "In the legend to Figure 4F the authors write "conditions were normalized to MEF KO". In the middle panel (Cebpb), however, fold induction of MEF KO was not set to "1" as has been done for the other two genes. Please correct or explain.

I made a mistake here, and was actually referring to Figure 3F (not 4F). Maybe the authors still want to correct that.

In line with this reviewer's comment and editorial request, in the legend to figure 3A we have now clarified that fold induction is set to 1

RE: Manuscript #E19-11-0628RRR
TITLE: "β-actin contributes to an open chromatin for activation of the adipogenic pioneer factor CEBPA during transcriptional reprograming"

Dear Dr. Percipalle:

Thank you for attending to the remaining comments from the reviewers. However, I am not yet satisfied that you have addressed the instructions from the MBoC Author Submission Checklist. I am including this list here for emphasis and I expect that it will be fully addressed in the next revision.

From the instructions:
The following information is available in all relevant figure legends (or has been placed in the Materials and Methods section to avoid excessively long legends):

1. The exact sample size (n) for each experimental group/condition, given as a number, not a range;
2. 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.);
3. A statement of how many times the experiment shown was replicated in the laboratory;
4. Definitions of statistical methods and measures:
a. Data from small samples (n<5), for which descriptive statistics are not appropriate, have been plotted as individual points.
b. Complex techniques are described in the Materials and Methods section (common tests, such as t test, simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests can be unambiguously identified by name only).
c. Tests are identified as one-sided or two-sided.
d. There are adjustments for multiple comparisons.
e. Statistical test results, e.g., P values, are given.
f. "Center values" are defined as median or mean.
g. Error bars are defined as s.d. or s.e.m. or c.i.

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Monitoring Editor
Molecular Biology of the Cell

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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Rebuttal letter, Manuscript #E19-11-0628RRR

As per editorial request, we have now included the information in all relevant figure legends of the revised manuscript. Where applicable this includes some of the following information

1. The exact sample size (n) for each experimental group/condition, given as a number, not a range;
2. 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.);
3. A statement of how many times the experiment shown was replicated in the laboratory;
4. Definitions of statistical methods and measures:
a. Data from small samples (n<5), for which descriptive statistics are not appropriate, have been plotted as individual points.
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Dear Dr. Percipalle:

Thanks for attending to the final issues with regard to the MBoC Author Submission Checklist. I am now pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Percipalle:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
