

Manuscript EMBO-2009-72898

## Essential role of p18Hamlet/SRCAP-mediated histone H2A.Z chromatin incorporation in muscle differentiation

Ana Cuadrado, Nadia Corrado, Eusebio Perdiguero, Vanesa Lafarga, Pura Muñoz-Canoves and Angel Nebreda

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### Review timeline:

Submission date:	19 October 2009
Editorial Decision:	19 November 2009
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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

19 November 2009

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Thank you for submitting your manuscript for consideration by the EMBO Journal. Your manuscript has now been seen by three referees and their comments to the authors are provided below.

As you can see, both referees #1 and 2 find the analysis interesting and suitable for publication in the EMBO Journal. However they also find that the analysis need to be extended and that in particular further support for that other SRCAP complex components, besides p18, are also involved in H2A.Z recruitment is needed. Referee #3, on the other hand, is not persuaded that we gain enough new understanding of how SRCAP functions in order to consider publication here. Given the comments provided by both referees #1 and 2, I will go with their recommendations. Therefore, should you be able to address their criticisms in full (and also keep in mind the specific points raised by referee #3), we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: <http://www.nature.com/emboj/about/process.html>

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 (Remarks to the Author):

This manuscript reports on a novel target of the p38 signalling - the SRCAP sub-unit, p18 hamlet - to the chromatin of muscle genes. The authors show that p18 hamlet is required for the accumulation of H2A.Z at the myogenin locus, an event that facilitates the transcription of myogenin at the onset of muscle differentiation.

This information increases our knowledge on the mechanism by which p38 kinases alpha and beta exert their pro-myogenic effect. The data convincingly support the author conclusions. As such, this manuscript appears adequate for publication in EMBO.

I encourage the authors to further improve the quality of the manuscript by resolving the following issues.

### Major

The authors should provide a definite demonstration that p38-mediated phosphorylation of p18 hamlet is required for deposition of H2A.Z at the myogenin locus during myogenic differentiation. To this end, I suggest that the p38 signaling is ectopically activated in myoblasts by introduction of the upstream activator MKK6EE. Co-expression of either wild type or phospho-mutant p18 hamlet should be used to determine if a p38 phosphorylation-resistant p18 hamlet impairs p38-dependent enrichment of H2A.Z at the myogenin promoter (using ChIP).

### Minor

Fig. 1C - why do the authors use immunoprecipitation>western blot, instead of standard western blot to determine the levels of p18 hamlet in response to p38 blockade?

Fig. 2 - the relative chromatin enrichment in the ChIP experiments shown in B and C should be determined by q-PCR in both experiments

Fig. 3C - reintroduction of p38 kinase alpha should be used to increase the biological significance of data reported in p38 alpha knock out satellite cells

Fig. 4F - the authors should show the expression levels of p18 hamlet by western blot in regenerating muscles. Also the immunofluorescence showing the co-expression of p18 hamlet and Pax7 should be done in muscle sections, to make sure that p18 hamlet is induced in satellite cells.

Referee #2 (Remarks to the Author):

This manuscript investigates the function of the p18 subunit of the SRCAP complex in muscle differentiation. The authors show that p18 and H2A.Z locate to a similar region of the myogenin promoter, that p18 is required for H2A.Z recruitment to this promoter and for transcriptional activation, and that phosphorylation of p18 by p38 kinase is required for recruitment by enhancing p18 binding to other components of SRCAP. It is concluded that SRCAP-mediated histone H2A.Z deposition is important in myogenesis-specific transcriptional activation. The manuscript is very interesting in that it implicates H2A.Z function in myogenic differentiation. However, a significant limitation is that the study is focused on p18 and does not adequately characterize the implied role of the remainder of the SRCAP complex in H2A.Z recruitment and differentiation. The authors should perform ChIP assays with antibodies against other SRCAP complex components to confirm recruitment of the complex to the promoter (Fig.3 A and C). Also, the Fig.5 results do not support the notion that the SRCAP complex, including p18, can bind to H2A.Z under the conditions used. Thus, H2A.Z binds only to ARP6 in Fig.5 C, but if the model in Fig.5 A is correct, H2A.Z should bind to YL-1. The authors should conduct the binding assay under conditions in which H2A.Z can bind to YL-1 or possibly after addition of DM to enhance activation of p18 binding to ARP6.

The authors also suggest, that p18 phosphorylation by p38 effects recruitment of H2A.Z (Fig. 3) and binding to other components of the SRCAP complex (Fig. 5). Actually, the data do show that H2A.Z recruitment is inhibited under conditions where p38 is inactivated or where the p18 mutant cannot bind to ARP6. However, as indicated above, additional data is needed to link these events to the SRCAP complex. For example, they should conduct ChIP assays with antibodies against other SRCAP components under conditions of p38 pathway inactivation and p18 knockdown. A more comprehensive analysis linking the function of p38/p18 to H2A.Z deposition and differentiation-related target gene activation through the complete SRCAP complex would greatly strengthen this potentially important study.

Referee #3 (Remarks to the Author):

This manuscript by Cuadrado and colleagues describe an essential role for a SRCAP-complex subunit (p18-Hamlet) in the incorporation of histone variant H2A.Z at genes involved in muscle differentiation. The authors further show that p18 and H2A.Z are essential for target gene expression and subsequent early muscle differentiation. Finally the authors show that phosphorylation of p18 by the p38 MAP kinase is essential for this pathway.

General appreciation:

The fact that SRCAP and H2A.Z are important for muscle differentiation is clearly important but the paper does not provide any significantly new insight into SRCAP/H2A.Z function. Much of what the authors describe has been conceptually documented in other reports.

Specific comments:

1- SRCAP does not control gene expression in yeast as stated in the abstract and Intro, this should be made clear. In addition, the Intro is misleading as far as SRCAP and p400 are concerned, both are orthologs of yeast Swr1, and p400 is completely omitted from this section. This appears to have been slightly clarified in the Discussion.

2- In several figures, there appears to have ChIP data presented as graphics and others simply presented as bands on agarose gels, why is that? Where qPCR has not been used, how can we have confidence that the PCR's are within linear range of quantification. This is important given that some results (e.g. Fig. 4D) do not appear very convincing. Also why change loading controls within the same figure (e.g. Fig. 1)?

3- In figure 2B, it is quite surprising that H3 levels are so much lower than H2A.Z? Also in the same panel, what does "relative binding ratio" mean? What are the % inputs?

4- In fig 3A, H2A.Z levels appear to increase as a function of time when visualized by a bar graph but when the same experiment is visualized on an agarose gel (Fig 3B), the increase is not very convincing.

5- It is unclear to me how Fig 5 fits into that story.

6- The authors show that p18 overexpression stimulates myogenin gene expression. That would imply that p18 is limiting to the SRCAP complex or that something else is going on- this should be addressed.

1st Revision - authors' response

16 March 2010

Revised manuscript EMBOJ-2009-72898. Response to Referees

Referee #1

*This manuscript reports on a novel target of the p38 signalling - the SRCAP sub-unit, p18 hamlet - to the chromatin of muscle genes. The authors show that p18 hamlet is required for the accumulation of H2A.Z at the myogenin locus, an event that facilitates the transcription of myogenin at the onset of muscle differentiation.*

*This information increases our knowledge on the mechanism by which p38 kinases alpha and beta exert their pro-myogenic effect. The data convincingly support the author conclusions. As such, this manuscript appears adequate for publication in EMBO.*

*I encourage the authors to further improve the quality of the manuscript by resolving the following issues.*

#### *Major*

*The authors should provide a definite demonstration that p38-mediated phosphorylation of p18 hamlet is required for deposition of H2A.Z at the myogenin locus during myogenic differentiation. To this end, I suggest that the p38 signaling is ectopically activated in myoblasts by introduction of the upstream activator MKK6EE. Co-expression of either wild type or phospho-mutant p18 hamlet should be used to determine if a p38 phosphorylation-resistant p18 hamlet impairs p38-dependent enrichment of H2A.Z at the myogenin promoter (using ChIP).*

To provide further support for the role of p38 MAPK phosphorylation on p18<sup>Hamlet</sup>-mediated H2A.Z deposition at chromatin, we have performed the suggested experiment co-expressing MKK6DD together with either p18<sup>Hamlet</sup> wt or the 4A mutant (Fig. 5B and 5C). Constitutively active MKK6DD and MKK6EE mutants have been extensively used to specifically activate the p38 MAPK pathway (Puri et al., 2000 ; Takekawa et al., 2000; Mace et al., 2005; Cuadrado et al., 2007). Our results indicate that in the presence of wt p18<sup>Hamlet</sup>, MKK6DD induces a significant increase in H2A.Z binding to the myogenin promoter. In contrast, co-expression of the non-phosphorylatable 4A mutant results in impaired H2A.Z recruitment, strongly supporting the importance of p38 MAPK-mediated p18<sup>Hamlet</sup> phosphorylation for the process.

#### *Minor*

*Fig. 1C - why do the authors use immunoprecipitation>western blot, instead of standard western blot to determine the levels of p18 hamlet in response to p38 blockade?*

We have used immunoprecipitation followed by western blot, instead of standard western blot, because the phosphorylation of endogenous p18<sup>Hamlet</sup> is difficult to detect with the available antibodies. We show in the Reviewer Fig 1 that total levels of p18<sup>Hamlet</sup> in response to p38 MAPK blockade can be detected by standard western blot as well.

*Fig. 2 - the relative chromatin enrichment in the ChIP experiments shown in B and C should be determined by q-PCR in both experiments*

As requested by the referee, we now show the relative chromatin enrichment in all the ChIP experiments by q-PCR analysis.

*Fig. 3C - reintroduction of p38 kinase alpha should be used to increase the biological significance of data reported in p38 alpha knock out satellite cells*

We have performed the indicated experiment and observed enhanced H2A.Z recruitment to the myogenin promoter upon re-expression of p38alpha in the p38alpha knockout primary myoblasts. Of note, p38alpha re-expression also induced myogenin expression (Supplementary Fig S2).

*Fig. 4F - the authors should show the expression levels of p18 hamlet by western blot in regenerating muscles. Also the immunofluorescence showing the co-expression of p18 hamlet and Pax7 should be done in muscle sections, to make sure that p18 hamlet is induced in satellite cells.*

We now show by western blot the enhanced expression levels of p18<sup>Hamlet</sup> in two independent samples of overloaded muscles -compare basal and ongoing myogenesis states (Fig 5 D, left panel). Regarding the co-expression experiments, we have failed in our attempts to perform the double immunofluorescences, most likely due to technical limitations of the available p18<sup>Hamlet</sup> antibodies.

#### *Referee #2*

*This manuscript investigates the function of the p18 subunit of the SRCAP complex in muscle differentiation. The authors show that p18 and H2A.Z locate to a similar region of the myogenin promoter, that p18 is required for H2A.Z recruitment to this promoter and for transcriptional activation, and that phosphorylation of p18 by p38 kinase is required for recruitment by enhancing*

*p18 binding to other components of SRCAP. It is concluded that SRCAP-mediated histone H2A.Z deposition is important in myogenesis-specific transcriptional activation. The manuscript is very interesting in that it implicates H2A.Z function in myogenic differentiation. However, a significant limitation is that the study is focused on p18 and does not adequately characterize the implied role of the remainder of the SRCAP complex in H2A.Z recruitment and differentiation. The authors should perform ChIP assays with antibodies against other SRCAP complex components to confirm recruitment of the complex to the promoter (Fig. 3 A and C).*

To strengthen the implication of the SRCAP complex in H2A.Z recruitment and muscle cell differentiation, we have performed ChIP assays with the catalytic subunit of the SRCAP complex (Fig. 6F). We observed that, as with p18<sup>Hamlet</sup> and H2A.Z, SRCAP can be detected at the myogenin promoter under proliferation conditions, and the binding significantly increases when cells are induced to differentiate. Moreover, the differentiation-induced SRCAP recruitment to chromatin is dependent on both p18<sup>Hamlet</sup> and p38 MAPK (Fig. 6F), supporting a role for this signalling pathway in SRCAP complex function. We also show that the downregulation of the SRCAP complex component YL-1, which has been reported to bind histones in yeast (Wu et al., 2005), impairs H2A.Z recruitment to the myogenin promoter (Fig. 8D).

*Also, the Fig. 5 results do not support the notion that the SRCAP complex, including p18, can bind to H2A.Z under the conditions used. Thus, H2A.Z binds only to ARP6 in Fig. 5 C, but if the model in Fig. 5 A is correct, H2A.Z should bind to YL-1. The authors should conduct the binding assay under conditions in which H2A.Z can bind to YL-1 or possibly after addition of DM to enhance activation of p18 binding to ARP6.*

The referee's comments concerning the binding of SRCAP subunits to H2A.Z under proliferation conditions are correct. To address this criticism, we have performed co-immunoprecipitations at several time-points during differentiation. We show now that according to the proposed model in yeast (see Fig. 6A), H2A.Z indeed binds to YL-1, the predicted histone binding subunit of the SRCAP complex, during myogenesis. However, the process appears to be regulated, so that binding of H2A.Z to YL-1 peaks at 8 h after the induction of differentiation and decreases 16 h later (Fig. 6E). Such dynamic behaviour suggests that H2A.Z chromatin recruitment is a transient and early event necessary to engage the muscle-specific transcription program.

*The authors also suggest, that p18 phosphorylation by p38 effects recruitment of H2A.Z (Fig. 3) and binding to other components of the SRCAP complex (Fig. 5). Actually, the data do show that H2A.Z recruitment is inhibited under conditions where p38 is inactivated or where the p18 mutant cannot bind to ARP6. However, as indicated above, additional data is needed to link these events to the SRCAP complex. For example, they should conduct ChIP assays with antibodies against other SRCAP components under conditions of p38 pathway inactivation and p18 knockdown. A more comprehensive analysis linking the function of p38/p18 to H2A.Z deposition and differentiation-related target gene activation through the complete SRCAP complex would greatly strengthen this potentially important study.*

We now show that binding of the SRCAP catalytic subunit to the myogenin promoter increases during myogenesis, and that either p38 MAPK inactivation (with SB203580) or p18<sup>Hamlet</sup> knockdown (with siRNA) impair SRCAP recruitment (Fig. 6F), further connecting p18<sup>Hamlet</sup> phosphorylation by p38 MAPK to H2A.Z recruitment and the SRCAP complex. A wider analysis of the SRCAP complex in differentiation-related target gene activation is limited by the lack of antibodies against other SRCAP subunits.

### Referee #3

*This manuscript by Cuadrado and colleagues describe an essential role for a SRCAP-complex subunit (p18-Hamlet) in the incorporation of histone variant H2A.Z at genes involved in muscle differentiation. The authors further show that p18 and H2A.Z are essential for target gene expression and subsequent early muscle differentiation. Finally the authors show that phosphorylation of p18 by the p38 MAP kinase is essential for this pathway.*

*General appreciation:*

*The fact that SRCAP and H2A.Z are important for muscle differentiation is clearly important but the paper does not provide any significantly new insight into SRCAP/H2A.Z function. Much of what the authors describe has been conceptually documented in other reports.*

We agree with the referee's statement that a major conclusion of our work is the important role of

SRCAP and H2A.Z in muscle differentiation. Regarding our contribution to how SRCAP/H2A.Z function, to our knowledge, this is the first report to show the regulation of this chromatin remodelling mechanism by the phosphorylation of one of the SRCAP complex components. We also demonstrate the implication of the p38 MAPK signalling pathway in this regulation.

*Specific comments:*

*1- SRCAP does not control gene expression in yeast as stated in the abstract and Intro, this should be made clear. In addition, the Intro is misleading as far as SRCAP and p400 are concerned, both are orthologs of yeast Swr1, and p400 is completely omitted from this section. This appears to have been slightly clarified in the Discussion.*

We have edited in the Abstract and Introduction the statement that SRCAP controls gene expression in yeast, which appears to be supported by some but not all publications. We now also mention in the Introduction that SRCAP and p400 are both related to yeast Swr1.

*2- In several figures, there appears to have ChIP data presented as graphics and others simply presented as bands on agarose gels, why is that? Where qPCR has not been used, how can we have confidence that the PCR's are within linear range of quantification. This is important given that some results (e.g. Fig. 4D) do not appear very convincing. Also why change loading controls within the same figure (e.g. Fig. 1)?*

We have now clarified both in the figures and figure legends that ChIP data are presented as graphics for qPCR results and as bands on agarose gels for semi-quantitative PCRs. Moreover, we now show qPCR analysis in all the ChIP experiments. Regarding Figure 1, we have always used tubulin as a loading control for total lysates, but this is not possible for immunoprecipitations where we use the IgG levels as a reference.

*3- In figure 2B, it is quite surprising that H3 levels are so much lower than H2A.Z? Also in the same panel, what does "relative binding ratio" mean? What are the % inputs?*

We do not think that H3 and H2A.Z levels can be compared in these experiments, as both histones may be immunoprecipitated with different efficiencies by the two antibodies used. Regarding the relative binding ratio, values were normalised to the respective input DNA and then referred to the binding of each histone under GM conditions, which was given the value of 1. In fact, if all values are referred to the binding of H2A.Z under GM conditions, the levels of H3 appear to be significantly higher than those of H2A.Z, as shown in the Reviewer Fig 2. However, as mentioned above, we think this is not really comparable. Nevertheless, we have now changed both the layout of the figure and the text in the figure legend to try to clarify this point.

*4- In fig 3A, H2A.Z levels appear to increase as a function of time when visualized by a bar graph but when the same experiment is visualized on an agarose gel (Fig 3B), the increase is not very convincing.*

In our opinion, both the bar graph (qPCR) and the agarose gel (semi-quantitative PCR) support the increase in H2A.Z levels as a function of time.

*5- It is unclear to me how Fig 5 fits into that story.*

The main point of Fig 5 (now Fig 6) was to analyse how the phosphorylation of p18<sup>Hamlet</sup> by p38 MAPK affected the integrity of the SRCAP complex. We have now added further data to show that H2A.Z binding to the SRCAP complex is regulated during myogenesis, and that the SRCAP catalytic subunit itself is also recruited to muscle specific genes in a manner dependent on both p38 MAPK and p18<sup>Hamlet</sup>.

*6- The authors show that p18 overexpression stimulates myogenin gene expression. That would imply that p18 is limiting to the SRCAP complex or that something else is going on- this should be addressed.*

Given that p18<sup>Hamlet</sup> is normally expressed at very low levels in proliferating cells (Cuadrado et al., 2007), we think that p18<sup>Hamlet</sup> may well be limiting to the SRCAP complex, or at least to the function of this chromatin remodelling complex during muscle differentiation.

References

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Wu, W.H., Alami, S., Luk, E., Wu, C.H., Sen, S., Mizuguchi, G., Wei, D. and Wu, C. (2005) Swc2 is a widely conserved H2AZ-binding module essential for ATP-dependent histone exchange. *Nat Struct Mol Biol*, 12, 1064-1071.

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Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees #1 and 2 to review the revised version and while referee #1 was available to do so, referee #2 was not. I also asked referee #1 to comment on your response to the concerns raised by referee #2. I have now received the comments back from referee #1 and as you can see below this referee finds the revised manuscript improved and supports publication here. I am therefore very pleased to proceed with the acceptance of the manuscript for publication here.

Thank you for submitting your interesting manuscript to the EMBO Journal.

Sincerely

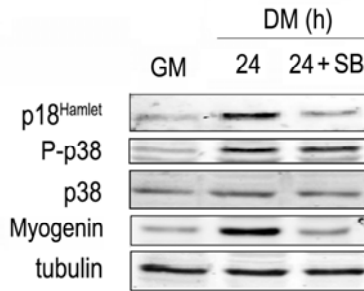
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#### REFeree REPORT

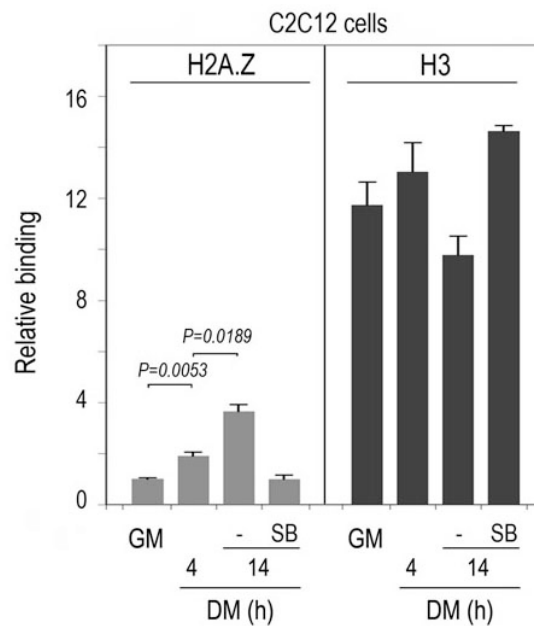
Referee #1

This manuscript, which was already interesting, has been greatly improved after the revision. All the concerns that I raised have been addressed. I therefore recommend publication.

## Referee-only Supplementary Material



**Reviewer Figure 1.** C2C12 myoblasts were cultured in GM or incubated in DM for 24 h, either in the absence or presence of SB203580 (SB). Total cell lysates were analysed by immunoblotting using the indicated antibodies.



**Reviewer Figure 2.** ChIP analysis of histones H2A.Z and H3 binding to the TATA box-containing region of the myogenin promoter in proliferating C2C12 myoblasts (GM) and at early times during muscle differentiation (DM) either in the presence or absence of SB203580 (SB). qPCR data are shown as means  $\pm$  SD of two independent experiments performed in triplicates. Histone binding values are normalised to the respective input DNA and are referred to the binding of H2A.Z under GM conditions, which is given the value of 1.