THE EMBO JOURNAL

Manuscript EMBO-2010-76785

C/EBPβ mediates tumor-induced ubiquitin ligase atrogin1/MAFbx upregulation and muscle wasting

Guohua Zhang, Bingwen Jin, Yi-Ping Li

Corresponding author: Yi-Ping Li, University of Texas Health Science Center at Houston

Review timeline:

Submission date:	15 December 2010
Editorial Decision:	31 January 2011
Revision received:	07 June 2011
Editorial Decision:	29 June 2011
Revision received:	19 July 2011
Accepted:	22 July 2011

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 31 January 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first of all apologise once again for the delay in getting back to you with a decision - we have only just received the report from the final referee. The comments of all three reviewers are enclosed below.

As you will see, all three referees recognise the interest in your work, but all also raise a significant number of concerns that would need to be addressed before we could consider publication. Most notable amongst these are the comments of referee 3 regarding the need for larger group sizes in the in vivo experiments. Extending the study accordingly would be an essential pre-requisite for potential publication here. Secondly, I would also highlight the concerns of referees 1 and 2 about the relative role of MuRF1 vs atrogin 1 in the regulation of MHC. Minimally, it would be essential to discuss this issue more extensively, but I would encourage you to follow the recommendations of these reviewers, and to look at a potential role of MuRF1 here. Finally, regarding referee 2's comments about the role - or lack of it - for FoxO in the regulation of atrogin 1, we would not insist on additional experiments here, but it would be necessary to tone down your claims that FoxO is not involved.

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

© European Molecular Biology Organization

visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. I realise that the experiments requested by the referees may require longer than three months (particularly given the need for in vivo work), and we can grant an extension (up to a maximum of six months) - please just let me know if you would like us to extend the deadline accordingly.

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 paper extends prior studies demonstrating the p38 pathway is required for MAFbx/Atrogin upregulation, and demonstrates a specific p38 and the downstream mediator, C/EBPbeta

Points:

Figure 1
The authors show that MuRF1 is not perturbed; however MHC is perturbed by LLC, via p38. This is surprising, since MHC has been shown to be a substrate of MuRF1, not MAFbx. It would be therefore of interest for the authors to see if LLC induced loss of MHC is blocked by knockdown of MAFbx vs MuRF1... to get an idea as to the causative E3 in this setting. Alternatively, perhaps p38 - C/EBPbeta is perturbing MHC transcription - that could also be assessed.

Figure 3B&3C
Was a dose-response done with SB? Also are there more specific controls, using phosho-specific antibodies on p38 phosphorylation sites, to prove that the SB is blocking p38 phosphorylation? The problem with figure 3C is that general p-Ser and p-Thr antibodies are used; it would be better to more directly demontrate inhibition of p38 with the SB protein, especially given how black-and-white the interaction data is in 3B.

Figure 4C
This is an important figure; could the controls please be shown? It is important to make sure there is similar expression of the other p38 molecules, and/or that the other expected substrates are phoshorylated by the various p38 molecules, as controls for the claimed specificity of p38beta.

Figure 5C
As mentioned above, the MHC data is surprising, since it has not been previously demonstrated that MHC is a substrate of MAFbx. So it would be of interest to see if the MHC loss is specific to MAFbx, as opposed to MURF1 (which several papers have demonstrated ubiquitinates MHC).

Very Minor:

1) Typo on page 4: PI3K, not IP3K

2) It is awkard to write "because that", which is used frequently in the manuscript; simply write "because". For example on page nine, the sentence should be "Because C/EBPbeta is not

© European Molecular Biology Organization

expressed..." not "Because that C/EBPbeta is not expressed..." and similarly with the other times "because that is used."

Referee #2 (Remarks to the Author):

General Comments

The authors presented data for a new role of C/EBPβ in upregulating atrogin1 in response to soluble factors secreted from Lewis lung carcinoma (LLC). The medium used to culture LLC cells induced phosphorylation of p38 and expression of atrogin1, but not MuRF1. Moreover, they found that p38 interacted with and phosphorylated C/EBPβ and atrogin1 promoter which contains the C/EBPβ binding site. Importantly, the authors also showed that inhibition of p38 or deficiency of C/EBPβ attenuated the enhanced mRNA levels of atrogin1 LLC-bearing mice. Despite these interesting data, this MS also contains several issues that need to be addressed, and a number of points are not very convincing in the present manuscript

Specific Points

The argument of FoxO inactivation was weak and merely based on a very transient increase (1-2 hours) in phosphorylation of FoxO by LLC-conditioned medium (Fig 1A), which probably includes multiple growth factors, and the total level of FoxO is lacking, which should be measured as the content of FoxO proteins can be increased by certain atrophying conditions. Since the authors have the atrogin1 promoter vector that contains consensus sites for both Foxo and C/EBP, the point that LLC-conditioned medium activates C/EBP but not FoxO can be definitively addressed by promoter activity assay when mutating one of these two sites.

Figure 1: In 1A, it seems that AKT was activated and FoxO3 was consequently inhibited. Although the authors showed phosphorylation of FoxO3, suggesting that it might not play any role in this setting (1A), why or how was AKT activated by LLC-medium? Is AKT phosphorylated by activated Ins/PI3K pathway? If this is the case, does it mean that LLC-mediated catabolic signaling by p38 was stronger than Ins/PI3K pathway? The authors mentioned this issue very briefly in the Discussion ("PI3K/AT/FoxO3 pathway is irrelevant to atrogin1 activation based on a previous study using C26-bearing mice (Ref. Penna et al., 2009)" page 13). However, a recent study using C26 tumor-bearing mice showed a critical role of FoxO3 in induction of atrogin1 and MuRF1 (Zhou et al., 2010), although the authors only mentioned expression of the two E3 ligases (page 13). This issue should be discussed further and more experiments need to be performed to rule out an effect of FoxO3. The authors claimed that MHC staining in panel D became thinner by LLC-medium. However, the images of stained MHC do not convincingly support the proposed catabolic activity of p38 at all, although the quantified values looked statistically significant. Do the authors have more convincing data? LLC-conditioned medium were used for a very long 72 hours in Fig 1D and was able to reduce the level of MHC proteins. It will be a necessary control to show whether the transcription of MHC is affected by this treatment.

Figure 2: The sequences of the promoters were included to provide a rationale to hypothesize regulation of C/EBPβ by p38. However, it's unclear why the authors assumed that C/EBPβ would be phosphorylated by p38. The authors just wrote "C/EBPβ is known to be phosphorylated at different sites by several protein kinases which regulate its transactivation activity" (the 7th line from bottom, page 7). Is C/EBP previously known to be phosphorylated by p38? If it is, it would be better to stress phosphorylation of C/EBPβ by p38. Moreover, I don't think that only sequence analysis for a putative binding site for a transcription factor can be an independent figure. Probably, it should be included in Fig. 4, because Fig 4 actually tested the potential activity of the C/EBPβ binding site.

Figure 3: Interaction and phosphorylation data look nice. However, does LLC-medium induce expression and phosphorylation of C/EBP ? It can be included in the other figure, e.g. figure 7.

Figure 4: This figure nicely showed requirement of both p38β and C/EBPβ for activation of the atrogin1 promoter. Do the authors know whether LLC-medium alone enhances the luciferase expression? In addition to overexpression of p38β and C/EBPβ (Fig. 4), it would be more relevant to other figures.

© European Molecular Biology Organization

Figure 5: I just wonder how specific is the C/EBPβ siRNA. Does it affect other isoforms of C/EBP? Some evidence and discussion would be useful.

Figure 6: Overall, these data nicely support the conclusion that activation of p38β is involved in muscle wasting induced by LLC. From D to H, comparison of values that support this conclusion do not seem consistent. For example, in D and E, the authors concluded wasting was less in SB-injected mice by comparing difference between white and black bars in either vehicle or SB. I wonder whether weight of muscles of LLC+SB was bigger than LLC+vehicle, because vehicle + PBS should be a real control in these experiments. This approach should be applied to other panels as well, as the authors did in panel C for real-time PCR data. In panel G, error bars in the graph are very small, however, heterogeneity of fiber sizes is very big as shown in the representative images, which is very normal. Can the authors explain how this was done other than providing a reference (Menconi et al., 2008)? Also, what do the error bars represent? SD? In Fig 6G and Fig 7F, the statistics for cross-section area were surprisingly tiny, considering the significant variation of CSA among fibers. How do the authors explain this surprising result?

Figure 7: As mentioned in the comments for Figure 3, does LLC influence C/EBPβ expression and phosphorylation in mice? Those data should be included in western blots of panel B. Also, a real control (PBS in WT) should be used for comparison of values.

It is interesting and important to show that inactivating p38 or C/EBP (chemically or genetically) can block LLC-induced loss of muscle mass (Fig 6 and 7), but giving exclusive emphasis as this manuscript did on the regulation of atrogin1 by C/EBP is not necessary. Although atrogin1 is one of the best markers for atrophy, activation of C/EBP may induce catabolism through multiple E3s and other components of UPS. Also, atrogin1 has not been connected to degrading MHC which seems to be a target for MURF1 (see Cohen et al. 2009), which was not induced by LCC as shown by the authors). How do the authors explain this apparent contradiction? The correlation between induction of atrogin1 and loss of MHC (Fig 5C) does not make much sense.

Minor: Published years in the references in the 2nd line of page 16 are omitted.

Referee #3 (Remarks to the Author):

Cancer induced weight loss and muscle wasting is a serious clinical problem. Currently, there is no satisfactory treatment. Therefore, it is very important to gain a better understanding of the molecular mechanisms of muscle wasting.
The authors investigate signaling pathways leading to muscle wasting induced by murine Lewis Lung carcinoma cells in an in vitro model (C2C12 cell myotubes) and in mouse models.
It had been shown previously that p38 induces atrogin1 expression and that atrogin1 mediates cancer-induced muscle wasting. Here, the role of specifically the p38b isoform was placed in the context of tumor cell induced muscle wasting and C/EBPb was identified as a mediator of p38-induced atrogin1 expression.
The authors data support previous reports that Murf1 is not relevant in cancer-induced loss of muscle mass.

The in vitro data using C2C12 cell myoblasts and myotubes are fairly convincing. However, the conclusions from in vivo experiments are confounded by too small group sizes and in the case of C/EBPb KO mice, the possible indirect effect of the host environment on the tumor cells. The most important finding is that the p38 inhibitor SB202190 rescues muscle fiber diameter (modestly) and reduces tyrosine release in tumor bearing mice.

1) General comment on data analysis.
Concerning the bargraphs it is not always clear which was the "control". For example, in Figure 1D is the SB/PBS bar set at 1 or compared to Vehicle/PBS? Usually, the control data that are set at "1" do not have an error bar?
In experiments with drug treatments, statistical P values should be given for all relevant comparisons, especially for LLC-conditioned medium or LLC-bearing mice with and without drug. For example, it is hard to believe that LAP+MKK6bE or +p38b has no effect on the pA-M

© European Molecular Biology Organization

construct, given the difference in the size of the bars (Fig.4B+D). Similarly, the decline in body weight in the SB group +/-LLC appears significant (Fig. 6D), especially if the difference in TA weight of the vehicle group is significant (Fig.6E). However, comparison of the two LLC groups (+/-SB) may confirm that SB rescue weight gain in LLC-bearing mice (Fig,6D). In Figure 6E, SB does not seem to have any effect on TA weight, when comparing LLC-bearing mice +/- SB!

2) Figure 1: The authors conclude that they ruled out Akt/FoxO signaling as an inducer of atrogin1. This is an overstatement. The authors analyzed FoxO1 and FoxO3. However, a recent paper showed that FoxO4 may be more important (PubMed ID 18701653). Furthermore, they did not confirm that SB treatment had no feed back effect on Akt activity. Therefore, "FoxOs" should be from the abstract and the conclusions should be phrased more cautiously.

3) Figure 1B: include expression of relevant components in the Input.
In the absence of an in vitro kinase assay, the conclusion that p38 directly phosphorylates C/EBPb should be phrased more cautiously.

4) Fig.1D: Include a negative control, i.e. a promoter that is not bound by C/EBPb.

5) Figure 2/3: By definition, a specific sequence cannot be a "consensus" sequence, though it may have homology with a consensus sequence. Therefore, "consensus" should be deleted and/or replaced with "putative" as appropriate. PubMed ID 8632009 provides a better designation for the C/EBP "consensus" of RTTGCGYAAY.
The C/EBP element is a putative site for all C/EBP family members. Some reporter/gene activity may be due to other C/EBPs. The authors should therefore provide a better rational for why they focused on C/EBPbeta, such as C/EBPb expression in muscle. The authors mention that C2C12 myoblasts do not express C/EBPbeta and continue to employ the LAP isoform of C/EBPb for overexpression studies. Therefore, it is pertinent to either cite or show the changes in C/EBPbeta isoform expression during C2C12 myotube differentiation.

6) Figure 1 and 3: Does expression of MKK6bE, p38 or SB treatment change the differentiation state of C2C12 myotubes?

7) Fig.3 etc.: It should be stated somewhere that the p38 antibodies do not differentiate between isoforms, or otherwise.

8) Fig.3C: Include Western analysis to confirm expression of all overexpressed proteins and/or a positive control for p38 isoform activity.

9) Fig.4: Basal activity may also be due to other C/EBPs.
GGGCCC is a poor choice for the C/EBP motif mutation because it creates a site for other transcription factors that may cause the activation of the pA-M construct.
See also general comment on data analysis above.

10) Fig.5A: Does silencing of C/EBPb affect C2C12 myotube differentiation?
Fig.5C: Include analysis of C/EBPb expression. The figure legends says atrogin1 and MHC were analyzed at "8h and 72h, respectively". In this case, each should have its own loading control as they could not have been done in the same extracts!

11) Fig.6A: In PubMed ID 21048967 no increase of p-p38 was observed in tumor bearing mice. This paper should be discussed, especially their finding that ERK-inhibitors reduced muscle wasting in tumor-bearing mice.

12) Fig.6B: The very large error bars for tumor sizes imply that some of the mice per group had almost no tumor. This could be due to graft rejection. Even though the LLC were derived from C57BL/6, there can be rejection within sublines of this strain. Since the focus of the study is the effect of the drug on muscle, the data could be improved by using larger group size and only animals whose tumors are more close in size.

13) Fig.6E/F. For a meaningful correlation to a clinical setting, the main question is if SB improves muscles in tumor-bearing mice. Fig.6E/F does not suggest that this is the case. Larger group sizes

© European Molecular Biology Organization

and using mice with comparable sized tumors may help.

14) Figure 7A. A previous report showed that growth of colon adenocarcinoma cell implants was impaired in C/EBPb-/- mice PubMed ID 19056928. The authors do not make the same observation with LLC, which should be discussed. Also, increasing the group size and disregarding mice with tumor cell rejection (since LLC and 129 are not compatible!) may very well lead to a different conclusion. This suggestion and given that C/EBPb null mice are leaner (not smaller, as stated on page 11; see PubMed ID 19056928), a host effect on tumor cell production of cachexia-inducing factors is possible. A muscle-specific deletion of a conditional C/EBPb allele (which is available, even on the C57BL/6 background) would be a much better approach. Since this is granted a large new effort, the present experiments could be repeated to increase group size and include only mice with similar sized tumors (even if this means treating mice for different times) for analysis of muscle tissue. Also, to make the model more clinically relevant, it would be better to begin SB treatment when tumors are palpable - not at the time of injection.

15) Figure 7D: LLC implants do not cause weight loss (cachexia) in C/EBPb null mice (Fig.7D)! This observation strongly suggests that host effects on tumor cells may be an indirect reason for the lack of muscle wasting in these mice, or mask the role of C/EBPb in muscle (muscle wasting is only a very small factor of total body weight loss).

Minor Points:

Specify mouse diet in the Methods. A 20-25% weight gain from age 8 to 10 weeks in the control mice seems like a lot.

In Figure 7D, there is a typo in the y-axis labels (0.15, should be 1.5)

There is a striking difference between the amount of details provided for the Methods and Figure Legends for Figures 1-5 (in vitro), and the lack of such details for Figures 6-7 (in vivo). For example, how was muscle fiber density quantified? How was TA mass determined? Give some description of the tyrosine release assy.

How were myotube diameters analyzed? How many fibers/ fields?

Gene silencing is not described in the Methods. Also, how is it timed with transfection of expression plasmids?

How was Wesetrn blot quantification done?

In several instances "Chromatin Immunoprecipitation" is abbreviated as "CHIP" instead of the correct "ChIP".

What was the medium for LLC cultures, which was used as control for conditioned medium?

Page 18: "National Institute of Cancer" should be "National Cancer Institute"

Provide references for the expression constructs.

Doyle A et al is a 2011 citation (year is either missing or wrong)

Include the official gene name Fbxo32 in the Abstract.

1st Revision - authors' response 07 June 2011

I am pleased to submit our revised manuscript EMBOJ-2010-76785R. In the revision, we responded to all comments by the reviewers with extensive new experiments. Point-to-point responses to reviews were detailed in a separate document. Here, let me respond to your comments.

1. Most notable amongst these are the comments of referee 3 regarding the need for larger group sizes in the in vivo experiments. Extending the study accordingly would be an essential pre-requisite for potential publication here.

We have completely redone the in vivo experiments. In experiments testing SB202190 effects, the starting time of drug administration has been changed to after the tumor became palpable. In experiments involving C/EBPβ knockout mice, we used mice in C57BL/6 background to replace mice in 129 background. Group size has been increased from 4 to 6. In addition, mice with tighter range of tumor size were used in all experiments. These measures significantly improved data quality.

2. Secondly, I would also highlight the concerns of referees 1 and 2 about the relative role of MuRF1 vs atrogin 1 in the regulation of MHC. Minimally, it would be essential to discuss this issue more extensively, but I would encourage you to follow the recommendations of these reviewers, and to look at a potential role of MuRF1 here.

It was known that atrogin1 upregulation reduces MHC level via degrading MyoD which regulates its expression. We added data that LCM suppresses MHC2B expression, which explains the loss of MHC.

3. Finally, regarding referee 2's comments about the role - or lack of it - for FoxO in the regulation of atrogin 1, we would not insist on additional experiments here, but it would be necessary to tone down your claims that FoxO is not involved.

We did a recommended definitive experiment to address this issue. By mutating the FoxO-responsive cis-elements in the atrogin1 promoter, we showed that the loss of these sites were inconsequential to LLC and p38 stimulation of the promoter activity. Thus, LLC and p38 upregulation of atrogin1 is independent of FoxO.

In addition to the above major issues, we responded to every comment by providing either new data or literature support. Therefore, I really hope this revision has met the high standards of your journal.

Reviewer 1

Q: Figure 1
The authors show that MuRF1 is not perturbed; however MHC is perturbed by LLC, via p38. This is surprising, since MHC has been shown to be a substrate of MuRF1, not MAFbx. It would be therefore of interest for the authors to see if LLC induced loss of MHC is blocked by knockdown of MAFbx vs MuRF1... to get an idea as to the causative E3 in this setting. Alternatively, perhaps p38 - C/EBPbeta is perturbing MHC transcription - that could also be assessed.

A: This issue is commented upon by all 3 reviewers. Reviewer 1 and 2 were surprised by this finding while Reviewer 3 concurred with our result. Previous studies indicated that atrogin-1/MAFbx upregulation induces MHC loss via an indirect mechanism by mediating MyoD degradation (PMID: 19319192). In fact, multiple studies on human patients reported loss of muscle mass and MHC with upregulation of atrogin1/MAFbx, but not MuRF1 (PMID: 16917114; PMID: 16507768). Furthermore, in a recent paper we showed that direct activation of p38 with MKK6 induces MHC loss without the upregulation of MuRF1 (PMID: 20826541). Thus, MuRF1 upregulation is not absolutely required for MHC loss. Acting upon this comment, we now show that LLC conditioned medium (LCM) down-regulates the mRNA of MHC2B (Figure S1), which explains the loss of MHC. The text has been revised to give more background and discussion on this issue.

Q: Figure 3B&3C
Was a dose-response done with SB? Also are there more specific controls, using phosho-specific antibodies on p38 phosphorylation sites, to prove that the SB is blocking p38 phosphorylation? The problem with figure 3C is that general p-Ser and p-Thr antibodies are used; it would be better to

© European Molecular Biology Organization

more directly demonstrate inhibition of p38 with the SB protein, especially given how black-and-white the interaction data is in 3B.

A: It is well established that SB202190 is p38-specific at the dose we used (10 μM), and at higher doses it loses the specificity. Therefore, it is a widely accepted practice that a single dose of 10 μM is used to inhibit p38. SB202190 is a small molecule inhibitor that prevents p38 from phosphorylating its substrates, not the phosphorylation of p38 itself. Therefore, a direct demonstration of inhibition of p38 phosphorylation by SB202190 using Western blot is not feasible. Our gain of functions assay using active p38 isoforms complements and supports the inhibitor assay (Figure 3C and S3).

Q: Figure 4C
This is an important figure; could the controls please be shown? It is important to make sure there is similar expression of the other p38 molecules, and/or that the other expected substrates are phoshorylated by the various p38 molecules, as controls for the claimed specificity of p38beta.

A: Control on the expression of the p38 isoforms and their phosphorylation of a p38 substrate (ATF2) are now shown (Figure S3). Verification data on the expression of LAP and MKK6 are also shown now (Figure S2).

Figure 5C
As mentioned above, the MHC data is surprising, since it has not been previously demonstrated that MHC is a substrate of MAFbx. So it would be of interest to see if the MHC loss is specific to MAFbx, as opposed to MURF1 (which several papers have demonstrated ubiquitinates MHC).

This is essentially the same question as the first one. Please see the answer above.

Additional corrections have been made in response to Very Minor concerns.

Reviewer 2

Q: The argument of FoxO inactivation was weak and merely based on a very transient increase (1-2 hours) in phosphorylation of FoxO by LLC-conditioned medium (Fig 1A), which probably includes multiple growth factors, and the total level of FoxO is lacking, which should be measured as the content of FoxO proteins can be increased by certain atrophying conditions. Since the authors have the atrogin1 promoter vector that contains consensus sites for both Foxo and C/EBP, the point that LLC-conditioned medium activates C/EBP but not FoxO can be definitively addressed by promoter activity assay when mutating one of these two sites.

A: We carried out the suggested definitive experiment and the results support our argument. Mutation of FoxO binding sites in the atrogin1/MAFbx promoter did not alter atrogin1/MAFbx upregulation by LCM (new Figure 3E). Thus, LCM induces atrogin1/MAFbx upregulation independent of FoxOs.

Q. Figure 1: In 1A, it seems that AKT was activated and FoxO3 was consequently inhibited. Although the authors showed phosphorylation of FoxO3, suggesting that it might not play any role in this setting (1A), why or how was AKT activated by LLC-medium? Is AKT phosphorylated by activated Ins/PI3K pathway? If this is the case, does it mean that LLC-mediated catabolic signaling by p38 was stronger than Ins/PI3K pathway? The authors mentioned this issue very briefly in the Discussion ("PI3K/AT/FoxO3 pathway is irrelevant to atrogin1 activation based on a previous study using C26-bearing mice (Ref. Penna et al., 2009)" page 13). However, a recent study using C26 tumor-bearing mice showed a critical role of FoxO3 in induction of atrogin1 and MuRF1 (Zhou et al., 2010), although the authors only mentioned expression of the two E3 ligases (page 13). This issue should be discussed further and more experiments need to be performed to rule out an effect of FoxO3. The authors claimed that MHC staining in panel D became thinner by LLC-medium. However, the images of stained MHC do not convincingly support the proposed catabolic activity of p38 at all, although the quantified values looked statistically significant. Do the authors have more convincing data? LLC-conditioned medium were used for a very long 72 hours in Fig 1D and was able to reduce the level of MHC proteins. It will be a necessary control to show whether the transcription of MHC is affected by this treatment.

© European Molecular Biology Organization

A: A previous study (PMID 18701653) demonstrated that TNF activates AKT via PI3K while upregulating atrogin-1, which is similar to our observation here. LCM could activate AKT similarly via PI3K. As indicated by this Reviewer, LLC may release growth factors, which would activate AKT via PI3K. As far as the paper by Zhou et al., 2010 is concerned, they did measure the AKT/FoxO signaling activity in Inhibin- KO Mice, but not in C26-bearing Mice (Figure 6D and E). So the reviewer's statement on this issue is incorrect. Our new data shown in Figure 2E clearly demonstrates the inconsequence of mutation of FoxO-responsive cis-elements on LLC upregulation of the atrogin1/MAFbx promoter. As requested, Figure 1D has been replaced by a more representative photo. MHC2B mRNA has been measured as recommended, which was down-regulated as the reviewer suspected (Figure S1).

Q: Figure 2: The sequences of the promoters were included to provide a rationale to hypothesize regulation of C/EBPb; by p38. However, it's unclear why the authors assumed that C/EBPb; would be phosphorylated by p38. The authors just wrote "C/EBPb; is known to be phosphorylated at different sites by several protein kinases which regulate its transactivation activity" (the 7th line from bottom, page 7). Is C/EBP previously known to be phosphorylated by p38? If it is, it would be better to stress phosphorylation of C/EBPb; by p38. Moreover, I don't think that only sequence analysis for a putative binding site for a transcription factor can be an independent figure. Probably, it should be included in Fig. 4, because Fig 4 actually tested the potential activity of the C/EBPb; binding site.

A: C/EBPβ is known to be activated by such inflammatory mediators as LPS, TNF , IL-6 and IL-1 that mediate muscle wasting. In addition, a previous study demonstrated that C/EBPβ is a p38 substrate in vitro (PMID: 9822687). It was our mistake not to provide the relevant background information, which has been added now. Figure 2 has been merged into Figure 3 (now Figure 2).

Q: Figure 3: Interaction and phosphorylation data look nice. However, does LLC-medium induce expression and phosphorylation of C/EBPb? It can be included in the other figure, e.g. figure 7.

A: Data on LCM induces increase in phosphorylation and expression of C/EBPβ are shown now (Figure 2F).

Q: Figure 4: This figure nicely showed requirement of both p38b; and C/EBPb; for activation of the atrogin1 promoter. Do the authors know whether LLC-medium alone enhances the luciferase expression? In addition to overexpression of p38b; and C/EBPb; (Fig. 4), it would be more relevant to other figures.

A: LCM does enhance the luciferase reporter expression, which is shown in new Figure 3E.

Q: Figure 5: I just wonder how specific is the C/EBPb; siRNA. Does it affect other isoforms of C/EBP? Some evidence and discussion would be useful.

A: We apologize for not providing the source information of the siRNA. The C/EBPβ siRNA was purchased from Dharmacon. The company sells isoform-specific C/EBP siRNAs and it does not affect other isoforms of C/EBP. A description of our siRNA procedure has been added.

Q: Figure 6: Overall, these data nicely support the conclusion that activation of p38b; is involved in muscle wasting induced by LLC. From D to H, comparison of values that support this conclusion do not seem consistent. For example, in D and E, the authors concluded wasting was less in SB-injected mice by comparing difference between white and black bars in either vehicle or SB. I wonder whether weight of muscles of LLC+SB was bigger than LLC+vehicle, because vehicle + PBS should be a real control in these experiments. This approach should be applied to other panels as well, as the authors did in panel C for real-time PCR data. In panel G, error bars in the graph are very small, however, heterogeneity of fiber sizes is very big as shown in the representative images, which is very normal. Can the authors explain how this was done other than providing a reference (Menconi et al., 2008)? Also, what do the error bars represent? SD? In Fig 6G and Fig 7F, the statistics for cross-section area were surprisingly tiny, considering the significant variation of CSA among fibers. How do the authors explain this surprising result?

© European Molecular Biology Organization

A: We have redone all in vivo experiments completely to increase sample size from 4 to 6 and used mice with tumor size within a tighter range, which improved data quality. Additional statistical analysis has been done as recommended for all figures with this type of comparisons. The photos for myofiber CSA have been replaced to include larger area of the muscle section to better represent the overall situation. CSA was measured by using the ImageJ software (the method of Menconi et al. was used to measure myotubes diameters). Approximately 500 myofibers within a muscle section were measured, which reduced SE (all our data are in SE).

Q: Figure 7: As mentioned in the comments for Figure 3, does LLC influence C/EBPb; expression and phosphorylation in mice? Those data should be included in western blots of panel B. Also, a real control (PBS in WT) should be used for comparison of values.

A: Data showing LLC induces increases in C/EBPβ expression and phosphorylation in mice has been added (now Figure 5B). We also discussed that C/EBPβ binding activity is dependent on its level of phosphorylation not expression.

Q: It is interesting and important to show that inactivating p38 or C/EBP (chemically or genetically) can block LLC-induced loss of muscle mass (Fig 6 and 7), but giving exclusive emphasis as this manuscript did on the regulation of atrogin1 by C/EBP is not necessary. Although atrogin1 is one of the best markers for atrophy, activation of C/EBP may induce catabolism through multiple E3s and other components of UPS. Also, atrogin1 has not been connected to degrading MHC which seems to be a target for MURF1 (see Cohen et al. 2009), which was not induced by LCC as shown by the authors). How do the authors explain this apparent contradiction? The correlation between induction of atrogin1 and loss of MHC (Fig 5C) does not make much sense.

A: Revision has been made to tone down the conclusion and acknowledge other possibilities as recommended. The issue on MHC being a recognized substrate of MuRF1 is discussed in detail in answer to Question 1 by Reviewer 1.
Reviewer 3

Q: 1) General comment on data analysis.
Concerning the bar graphs it is not always clear which was the "control". For example, in Figure 1D is the SB/PBS bar set at 1 or compared to Vehicle/PBS? Usually, the control data that are set at "1" do not have an error bar? In experiments with drug treatments, statistical P values should be given for all relevant comparisons, especially for LLC-conditioned medium or LLC-bearing mice with and without drug. For example, it is hard to believe that LAP+MKK6bE or +p38b has no effect on the pA-M construct, given the difference in the size of the bars (Fig.4B+D). Similarly, the decline in body weight in the SB group +/-LLC appears significant (Fig. 6D), especially if the difference in TA weight of the vehicle group is significant (Fig.6E). However, comparison of the two LLC groups (+/-SB) may confirm that SB rescue weight gain in LLC-bearing mice (Fig,6D). In Figure 6E, SB does not seem to have any effect on TA weight, when comparing LLC-bearing mice +/- SB!

A: We have increased the sample size and tightened tumor volume range in all in vivo experiments, which helped resolve the data quality and statistical issues. In Figure 1D the SB/PBS bar was a comparison to Vehicle/PBS. Control data are now set at 1 without error bars as recommended. Previously, we averaged the absolute value of control data to obtain the error bars.

Q: 2) Figure 1: The authors conclude that they ruled out Akt/FoxO signaling as an inducer of atrogin1. This is an overstatement. The authors analyzed FoxO1 and FoxO3. However, a recent paper showed that FoxO4 may be more important (PubMed ID 18701653). Furthermore, they did not confirm that SB treatment had no feed back effect on Akt activity. Therefore, "FoxOs" should be from the abstract and the conclusions should be phrased more cautiously.

A: PubMed ID 18701653 demonstrated that TNF activates AKT, and AKT signaling does not mediate TNF effect on atrogin1. Instead, TNF upregulates atrogin1 via upregulating the expression of Foxo4 (not the activity), which is independent of AKT-Foxo1/3 signaling. These data suggest that TNF effect on atrogin1 is not dependent on AKT regulation of Foxo1/3 phosphorylation state, therefore, are consistent with our data. Our new data from mutation of Foxo binding sites shown in new Figure 3 definitively ruled out Foxo in mediating atrogin1 upregulation by p38 or LLC.

© European Molecular Biology Organization

Q: 3) Figure 1B: include expression of relevant components in the Input. In the absence of an in vitro kinase assay, the conclusion that p38 directly phosphorylates C/EBPb should be phrased more cautiously.

A: We think "Figure 1B" is meant to be Figure3B by the reviewer regarding p38 kinase activity. The input for Figure 3B (now Figure 2C) is shown in Figure 2B now. Equal amount of proteins from the three treatments were used in immunoprecipitation. To demonstrate the effect of p38 activation and inhibition, we show the phosphorylation state of Thr-188 of C/EBPβ in Figure 2C, although it is only one of the many possible phosphorylation sites in C/EBP . As mentioned above, we mistakenly left out a previous report that C/EBPβ is a p38 substrate in vitro (PMID 9822687). In consideration of this information and our data presented here, C/EBPβ is likely a p38 substrate. Nevertheless, the related conclusion has been toned down to address this reviewer's concern.

Q: 4) Fig.1D: Include a negative control, i.e. a promoter that is not bound by C/EBPb.

A: Since Fig.1D does not contain any promoter study, we guess this is a typo meant to be Fig. 4D. Construct pB we used wis exactly such control the reviewer wants.

Q: 5) Figure 2/3: By definition, a specific sequence cannot be a "consensus" sequence, though it may have homology with a consensus sequence. Therefore, "consensus" should be deleted and/or replaced with "putative" as appropriate. PubMed ID 8632009 provides a better designation for the C/EBP "consensus" of RTTGCGYAAY.
The C/EBP element is a putative site for all C/EBP family members. Some reporter/gene activity may be due to other C/EBPs. The authors should therefore provide a better rational for why they focused on C/EBP , such as C/EBPβ expression in muscle. The authors mention that C2C12 myoblasts do not express C/EBPβ and continue to employ the LAP isoform of C/EBPβ for overexpression studies. Therefore, it is pertinent to either cite or show the changes in C/EBPβ isoform expression during C2C12 myotube differentiation.

A: We changed the word "consensus" to "putative" as recommended. The software at http://www.cbil.upenn.edu/cgi-bin/tess that we used to identify the binding site is able to differentiate the binding sites of C/EBP isoforms, and called this motif (TTGTGCAA) in the atrogin1 promoter specifically as a potential C/EBP -binding site. Our understanding on this issue is that although some common features allowed the proposal of a consensus-binding sequence for , and isoforms (RTTGCGYAAY) in PMID 8632009, C/EBPβ has its preferential and distinguishable binding sequence. Expression of C/EBPβ in differentiated C2C12 myotubes and in muscle has been shown previously (PMID: 16909111). We realized that our statement "C/EBPβ is not expressed in undifferentiated C2C12 myoblasts" (line 8, page 9) gave an impression that muscle cells do not normally express C/EBP , and modified this sentence to: "C/EBPβ is not expressed before C2C12 myoblasts are differentiated".

Q: 6) Figure 1 and 3: Does expression of MKK6bE, p38 or SB treatment change the differentiation state of C2C12 myotubes?

A: MKK6bE expression is mediated by adenovirus and the infection was initiated after the formation of myotubes. SB treatment was also given after the formation of myotubes. Therefore, differentiation was not affected.

Q: 7) Fig.3 etc.: It should be stated somewhere that the p38 antibodies do not differentiate between isoforms, or otherwise.

A: Yes, the antibody we used does not differentiate between p38 isoforms. This statement has been added.

Q: 8) Fig.3C: Include Western analysis to confirm expression of all overexpressed proteins and/or a positive control for p38 isoform activity.

A: Again, we think Fig. 3C was meant to be Fig. 4C, because Figure 3C did not involve p38 isoform activity. The recommended controls are shown now (Figure S2 and S3).

© European Molecular Biology Organization

Q: 9) Fig.4: Basal activity may also be due to other C/EBPs. GGGCCC is a poor choice for the C/EBP motif mutation because it creates a site for other transcription factors that may cause the activation of the pA-M construct. See also general comment on data analysis above.

A: For the possible involvement of other C/EBPs, see response to Question 5 above. We agree that mutation to GGGCCC may not be the best choice. But, there is always the possibility that mutation can create a binding site for some known or unknown transcription factors. The bottom line is that the mutation compromised the capacity of this cis element in enhancing the promoter activity.

Q: 10) Fig.5A: Does silencing of C/EBPb affect C2C12 myotube differentiation?
Fig.5C: Include analysis of C/EBPb expression. The figure legends says atrogin1 and MHC were analyzed at "8h and 72h, respectively". In this case, each should have its own loading control as they could not have been done in the same extracts!

A: Silencing of C/EBPβ did not affect C2C12 myotube formation in our experiments. Analysis of C/EBPβ expression after siRNA knockdown was shown in Figure 5A (now Figure 4A). Loading control at 8 h and 72 h are now shown individually in revised figure.

Q: 11) Fig.6A: In PubMed ID 21048967 no increase of p-p38 was observed in tumor bearing mice. This paper should be discussed, especially their finding that ERK-inhibitors reduced muscle wasting in tumor-bearing mice.

A: Discussion on this issue has been added (page 18).

Q: 12) Fig.6B: The very large error bars for tumor sizes imply that some of the mice per group had almost no tumor. This could be due to graft rejection. Even though the LLC were derived from C57BL/6, there can be rejection within sublines of this strain. Since the focus of the study is the effect of the drug on muscle, the data could be improved by using larger group size and only animals whose tumors are more close in size.

A: The experiment has been redone totally by tightening tumor size range to 1 - 1.5 cm and increasing sample size. In addition, we postponed the administration of SB till tumor is palpable. These measures resolved this issue.

Q: 13) Fig.6E/F. For a meaningful correlation to a clinical setting, the main question is if SB improves muscles in tumor-bearing mice. Fig.6E/F does not suggest that this is the case. Larger group sizes and using mice with comparable sized tumors may help.

A: Again, the new experiment resolved this issue.

Q: 14) Figure 7A. A previous report showed that growth of colon adenocarcinoma cell implants was impaired in C/EBPb-/- mice PubMed ID 19056928. The authors do not make the same observation with LLC, which should be discussed. Also, increasing the group size and disregarding mice with tumor cell rejection (since LLC and 129 are not compatible!) may very well lead to a different conclusion. This suggestion and given that C/EBPb null mice are leaner (not smaller, as stated on page 11; see PubMed ID 19056928), a host effect on tumor cell production of cachexia-inducing factors is possible. A muscle-specific deletion of a conditional C/EBPb allele (which is available, even on the C57BL/6 background) would be a much better approach. Since this is granted a large new effort, the present experiments could be repeated to increase group size and include only mice with similar sized tumors (even if this means treating mice for different times) for analysis of muscle tissue. Also, to make the model more clinically relevant, it would be better to begin SB treatment when tumors are palpable - not at the time of injection.

A: Discussion on data in PubMed ID 19056928 has been added. In addition to this issue, PubMed ID 19056928 showed that C/EBPβ contributes to endocrine expression of IGF-1, leptin and insulin, which are decreased in C/EBP -/- mice. We observed lower net body weight gain, smaller muscle mass and higher muscle proteolysis in C/EBP -/- mice as one would expect due to FoxO activation by weaker IGF-1 and insulin signaling. Notwithstanding, C/EBP -/- mice are resistant to LLC-induced atrogin-1 upregulation and muscle wasting, which supports a predominant role for C/EBPβ in mediating muscle catabolism in this model. The strain compatibility issue has been addressed by

© European Molecular Biology Organization

replacing 129 C/EBP -/- mice with C57/B6 C/EBP -/- mice. Experiment with SB treatment starting at the time tumors are palpable has been done to replace the old data.

Q: 15) Figure 7D: LLC implants do not cause weight loss (cachexia) in C/EBPβ null mice (Fig.7D)! This observation strongly suggests that host effects on tumor cells may be an indirect reason for the lack of muscle wasting in these mice, or mask the role of C/EBPβ in muscle (muscle wasting is only a very small factor of total body weight loss).

A: Figure 7D was about the muscle mass loss of TA in C/EBP -/- mice, not body weight loss (it was normalized to body weight). Total body weight change was shown in Figure 7C. It is difficult to assess whether tumor factors or host effects are responsible for muscle loss in vivo. Our data from myotubes suggest that tumor factors are critical in this model. On the other hand, it does not rule out host effects completely.

2nd Editorial Decision 29 June 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76785R. It has now been seen again by referees 2 and 3, whose comments are enclosed below. Both find the manuscript to be substantially improved, but both also raise a number of remaining issues that need to be resolved before we can consider publication here. I would say that we are still positive about publishing your work, but it is critical that these concerns are addressed first.

A few points regarding these reports:
- Referee 2 questions the presence/absence of error bars for your controls: the correct approach here is not to have error bars in cases where you have normalised the control samples. In these cases, it is of course critical that there not be significant variability between control experiments, and some statement to this effect would be valuable. In general, it is important to state explicitly in the figure legends how the experiments were normalised, as well as the number of replicates for each experiment (this is currently missing).
- Regarding the concerns still raised as to whether or not FoxO has a function here, I reiterate that we do not see the need for further experiments, but referee 3 does raise an important point that you need to be more specific as to which FoxOs you have looked at, and to acknowledge the possibility that some level of FoxO-dependent regulation may be relevant.
- Referee 2 still finds that you need to provide direct evidence for the C/EBPbeta siRNAs used here. I agree that this is important: given the well-known off-target effects of siRNAs, showing that the other C/EBP isoforms are not affected would strengthen the evidence that it is specifically C/EBPbeta that is important here. Do you have or can you easily obtain antibodies to test this? If you foresee any difficulty in doing this control experiment, please let me know.
- Please provide some evidence that ectopic expression of MKK6bE or p38, SB treatment or C/EBPbeta knockdown does not affect myoblast differentiation (as requested by referee 3).
- The recent publication by the Hasselgren lab should be cited and discussed.

Obviously given this recent publication, we would like to proceed as quickly as possible with publication of your work, assuming the remaining issues can be resolved, but I do find these to be important concerns that need to be addressed. I hope this can be done quickly, but please let me know if you expect any problems with this.

Otherwise, I look forward to receiving your revision soon.

Best wishes,

Editor
The EMBO Journal

———

© European Molecular Biology Organization

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

This paper presents several important findings about cancer cachexia, and overall, the revised manuscript is significantly improved. The additional data strengthen their novel conclusions and they tried to answer the reviewers' many concerns. Nevertheless, there are critical issues and questions remaining.

Although all the repeated values should have error bars, it is hard to believe that there is no error bar in Figures 1 to 6 for the control value, e.g. Fig. 1C for Atrogin1 mRNA level, PBS+Vehicle, similar in 1D, 1E, 2B, 2C,2D, 2E (Control for pA), etc. This must be due to the authors' misunderstanding of Reviewer 3's first comment. If this is the case, I am not sure how the authors could collect data and prepare representative figures.

Several general problems-The authors need to be more cautious in use of language. 1) These findings are intriguing for this model of cancer cachexia, but it seems dangerous to generalize to other cancers since other studies have suggested other mediators (Il-6, activin) and have described cachexia models where MuRF1 is induced. 2) They should clearly distinguish throughout the effects of conditioned medium on myotubes and effects of the tumor on muscle wasting in vivo. Both the tumor and the target muscle cells are likely to differ. 3) It is misleading to refer to Myosin HC as "myofibrillar proteins" since myotubes do not contain real myofibrils. Also, myosin is at most only a few percent of proteins and changes in MHC can't account for a smaller cytosol (p. 6-7). Also, these effects are through changes in mRNA, not myosin degradation.

The followings are unanswered questions, and some were also asked by other reviewers.

Figure 1: In 1A, FoxO3 is inhibited by activated AKT in LCM, and the obvious question was why or how the enhanced AKT does not inhibit Atrogin1 expression. The authors answered by referring to a paper from Reid's lab (Moylan et al., 2008), which does not provide any clear answer to this point. The only common observation between Moylan et al., and the authors' result is that activation of AKT didn't inhibit induction of Atrogin1. That report in fact showed that FoxO4 mediates Atrogin1 expression by TNF. If the authors claim that the Reid paper is consistent with their result, can the author exclude the effect of TNF and FoxO4 on atrogin1 expression in their LCM experiments? This point has been also questioned by reviewer 3.

In 1E, the image of myotubes used to analyze the effect of SB to inhibition is not clear at all. Moreover, the authors measured the diameter of myotubes. What is the diameter of the very long myotubes? This is a very subjective method to measure the size of cultured myotubes. Therefore, I don't know how authors could draw the graph under the image.

Figure 3: The authors were previously asked whether LLC alone could activate the pA reporter gene (in new figure 3A). In their rebuttal, the authors said that in Figure 3E, LCM does enhance the reporter expression (page 3 in rebuttal). However, there is no activation of the pA reporter by LCM alone. Where is that result? They should be more careful in answering the reviewers' comments.

Figure 4: They were also asked about the specificity of siRNA, if it's specific to C/EBPβ or other isoforms. The authors said the company sells the siRNA specific to C/EBP isoform. All commercial companies claim specificity for their products often without any evidence. Regardless of the company's advertisement, the authors should test the sequence specificity themselves by Western blots of isoforms using specific antibodies. If siRNA is not specific or the antibodies to C/EBP isoforms are not available, that would raise a serious question about specificity, as the other reviewer asked.

Figure 5 and 6: The authors repeated all the in vivo experiments, which are very reassuring. However, as commented in the last review, it is not still clear how the authors could obtain such tiny error bar, although they measured 500 fibers. The graph must represent the mean value of the measurement. Measured values of cross-sectional area should be plotted as frequency histogram, especially since fibers vary widely in size and in tending to atrophy often do not fit a normal

© European Molecular Biology Organization

distribution.

Referee #3 (Remarks to the Author):

The authors have made many adjustments and significant improvements to the data and manuscript. The following issues still require attention:

Repsonse to Q#2: Mutation of a promoter-reporter construct is insufficient to rule out endogenous gene regulation by any Foxo. In contrast to the authors' statement, PubMed ID#18701653 employs FOXO4 silencing to show the role of FOXO4 activity. The authors should specify at each occasion, which Foxo is being talked about. For example, it is incorrect to state that Moylan et al, showed that atrogin is upregulated in a Foxo-independent manner (page 4). Similarly, they should specify in the abstract which Foxo(s) they ruled out.

Response to Q#3: The figure legend should be explicit about 2B representing the input for the IP in 2C.

Response to Q#4: The point of using a different promoter as an additional negative control, was to provide evidence that the residual induction of pB by LAP+kiase may be unspecific. Now that the authors provide better description of the statistics and the residual activation is apparently not statistically significant, this control is no longer necessary.

Response to Q#5: As an explanatory note, I want to reiterate that no software - only appropriate experiments - can determine which specific C/EBP isoforms bind to a given promoter element. Software analysis results are misleading. They give isoform-specific labels, because the sequence may be identical to a sequence that was previously described as binding one isoform. Such data do not exclude that a different isoform can bind the same site in the same or maybe in the context of a different promoter. PubMed 8632009 identified "preferential" sequences for C/EBPbeta binding in vitro. In vivo, binding preferences are strongly influenced by cooperation with other factors that also bind the promoter.

Response to Q#6 does not address the question. Just because expression of the ectopic proteins or SB was initiated after differentiation does not exclude the possibility that they affect the differentiation state, which may be flexible if not reversible - especially in established cell lines. The authors should mention some evidence that the cells remain differentiated.

Response to Q#9: As explained above, the authors' response with regard to excluding other isoforms based on sequence is not valid. My earlier comments were motivated by the apparent activation of pA by kinases in the absence of LAP. This seems not to be the case after addition of statistical details.

Response to Q#10: The authors respond that C/EBPbeta-silencing does not affect myoblast differentiation. How did they arrive at this conclusion? Evidence should be included.
If panel 4A shows extracts from the same cells used in 5C, this should be stated explicitly. Otherwise, the C/EBPbeta control needs to be shown in 5C as it is customary for transient knockdown experiments.

Response to Q#15: The authors should include the possibility of host effects in their Discussion.

Additional comments:

1) Figure S2: Expression of C/EBPbeta appears enhanced in the last two lanes when assessed by the C/EBPbeta antibody, but not by anti- Flag. How do the authors explain this discrepancy?

2) In March, the following paper came on-line:

C/EBPβ regulates dexamethasone-induced muscle cell atrophy and expression of atrogin-1 and MuRF1.
Gonnella P, Alamdari N, Tizio S, Aversa Z, Petkova V, Hasselgren PO.

© European Molecular Biology Organization

J Cell Biochem. 2011 Jul;112(7):1737-48. doi: 10.1002/jcb.23093.
PMID: 21381078

It is appropriate to cite this paper in a relevant context.

2nd Revision - authors' response 19 July 2011

Thank you for your guidance on the revision. Additional experiments have been done to address referees' concerns. We are resubmitting the revised manuscript and our response to the referees. Below is our point-by-point response to your instructions.

We thank the insightful comments by the reviewers. The following is point-by-point response to the comments.

Referee #2 (Remarks to the Author):

Q: Although all the repeated values should have error bars, it is hard to believe that there is no error bar in Figures 1 to 6 for the control value, e.g. Fig. 1C for Atrogin1 mRNA level, PBS+Vehicle, similar in 1D, 1E, 2B, 2C,2D, 2E (Control for pA), etc. This must be due to the authors' misunderstanding of Reviewer 3's first comment. If this is the case, I am not sure how the authors could collect data and prepare representative figures.

A: Per editor's instruction we do not show error bars in cases where the control samples are normalized. This approach is stated now in Materials and Methods (page 28). Number of replicates is indicated in each figure or legend (n=x).

Q: Several general problems-The authors need to be more cautious in use of language. 1) These findings are intriguing for this model of cancer cachexia, but it seems dangerous to generalize to other cancers since other studies have suggested other mediators (Il-6, activin) and have described cachexia models where MuRF1 is induced. 2) They should clearly distinguish throughout the effects of conditioned medium on myotubes and effects of the tumor on muscle wasting in vivo. Both the tumor and the target muscle cells are likely to differ. 3) It is misleading to refer to Myosin HC as "myofibrillar proteins" since myotubes do not contain real myofibrils. Also, myosin is at most only a few percent of proteins and changes in MHC can't account for a smaller cytosol (p. 6-7). Also, these effects are through changes in mRNA, not myosin degradation.

A: Revisions have been made in response to 1) in abstract and on page 20 to focus on the LLC model, 2) on page 15 to distinguish the in vitro and in vivo effects, and 3) on page 6 and 16 to remove the association of MHC to myofibrillar proteins.

Q: The followings are unanswered questions, and some were also asked by other reviewers.

Figure 1: In 1A, FoxO3 is inhibited by activated AKT in LCM, and the obvious question was why or how the enhanced AKT does not inhibit Atrogin1 expression. The authors answered by referring to a paper from Reid's lab (Moylan et al., 2008), which does not provide any clear answer to this point. The only common observation between Moylan et al., and the authors' result is that activation of AKT didn't inhibit induction of Atrogin1. That report in fact showed that FoxO4 mediates Atrogin1 expression by TNF. If the authors claim that the Reid paper is consistent with their result, can the author exclude the effect of TNF and FoxO4 on atrogin1 expression in their LCM experiments? This point has been also questioned by reviewer 3.

A: You are right. We corrected the mistake by explicitly indicating that the Reid paper identified FoxO4 involvement and that it is FoxO1/3 we are referring to in this study, not all FoxOs. Yes, the common finding with the Reid group paper is that atrogin1 is upregulated while AKT is activated, although the stimuli in the two studies are different. Our data indicate a predominant role of

© European Molecular Biology Organization

p38beta-C/EBPbeta signaling in the regulation of the atrogin1 gene in the context of LLC tumor-induced muscle wasting, which overrides AKT. We did not examine if TNF and Foxo4 mediate LCM effect, because we did not consider TNF a primary mediator of LCM effect observed in this study as discussed on page 20 and 21.

Q: In 1E, the image of myotubes used to analyze the effect of SB to inhibition is not clear at all. Moreover, the authors measured the diameter of myotubes. What is the diameter of the very long myotubes? This is a very subjective method to measure the size of cultured myotubes. Therefore, I don't know how authors could draw the graph under the image.

A: We replaced the photo of SB/LCM treated myotubes with one that better represent the average effect of SB. The method of measuring myotube diameters was first published by Menconi et al. (PMID: 18615595). We adopted the method in a recently published paper (PMID: 20826541). Other investigators also used this method (PMID: 19955483). It involves the measurement of a single myotube at 3 different points and the average is used.

Q: Figure 3: The authors were previously asked whether LLC alone could activate the pA reporter gene (in new figure 3A). In their rebuttal, the authors said that in Figure 3E, LCM does enhance the reporter expression (page 3 in rebuttal). However, there is no activation of the pA reporter by LCM alone. Where is that result? They should be more careful in answering the reviewers' comments.

A: We meant that just like MKK6 and p38beta, LCM is capable of stimulating the promoter activity via C/EBPbeta (LAP). We apologize for not making the statement more accurate.

Q: Figure 4: They were also asked about the specificity of siRNA, if it's specific to C/EBPβ; or other isoforms. The authors said the company sells the siRNA specific to C/EBP isoform. All commercial companies claim specificity for their products often without any evidence. Regardless of the company's advertisement, the authors should test the sequence specificity themselves by Western blots of isoforms using specific antibodies. If siRNA is not specific or the antibodies to C/EBP isoforms are not available, that would raise a serious question about specificity, as the other reviewer asked.

A: We obtained the sequences of the siRNA pool from the company. A blast analysis found that the 4 siRNA sequences were indeed specific for C/EBPbeta. We verified the specificity by comparing the siRNA effect on C/EBPalpha level, which was not affected (Figure S4). In addition, we were able to rescue atrogin1 expression in myotubes where C/EBPbeta had been knocked down by overexpressing LAP (Figure S4). These lines of evidence support the specificity of the siRNA of C/EBPbeta.

Q: Figure 5 and 6: The authors repeated all the in vivo experiments, which are very reassuring. However, as commented in the last review, it is not still clear how the authors could obtain such tiny error bar, although they measured 500 fibers. The graph must represent the mean value of the measurement. Measured values of cross-sectional area should be plotted as frequency histogram, especially since fibers vary widely in size and in tending to atrophy often do not fit a normal distribution.

A: The data has now been re-plotted as recommended.

Referee #3 (Remarks to the Author):

Q: Response to Q#2: Mutation of a promoter-reporter construct is insufficient to rule out endogenous gene regulation by any Foxo. In contrast to the authors' statement, PubMed ID#18701653 employs FOXO4 silencing to show the role of FOXO4 activity. The authors should specify at each occasion, which Foxo is being talked about. For example, it is incorrect to state that Moylan et al, showed that atrogin is upregulated in a Foxo-independent manner (page 4). Similarly, they should specify in the abstract which Foxo(s) they ruled out.

A: You are right. We revised the abstract and text to explicitly indicate that FoxO1/3, not all FoxOs, are involved here in every relevant place.

© European Molecular Biology Organization

Q: Response to Q#3: The figure legend should be explicit about 2B representing the input for the IP in 2C.

A: Revised accordingly.

Q: Response to Q#4: The point of using a different promoter as an additional negative control, was to provide evidence that the residual induction of pB by LAP+kiase may be unspecific. Now that the authors provide better description of the statistics and the residual activation is apparently not statistically significant, this control is no longer necessary.

A: Okay.

Q: Response to Q#5: As an explanatory note, I want to reiterate that no software - only appropriate experiments - can determine which specific C/EBP isoforms bind to a given promoter element. Software analysis results are misleading. They give isoform-specific labels, because the sequence may be identical to a sequence that was previously described as binding one isoform. Such data do not exclude that a different isoform can bind the same site in the same or maybe in the context of a different promoter. PubMed 8632009 identified "preferential" sequences for C/EBPbeta binding in vitro. In vivo, binding preferences are strongly influenced by cooperation with other factors that also bind the promoter.

A: We concur. That is why we conducted multiple experiments, in vitro and in vivo, to verify the involvement of C/EBPbeta in mediating atrogin1 upregulation and muscle wasting in this model.

Q*: Response to Q#6 does not address the question. Just because expression of the ectopic proteins or SB was initiated after differentiation does not exclude the possibility that they affect the differentiation state, which may be flexible if not reversible - especially in established cell lines. The authors should mention some evidence that the cells remain differentiated.*

A: We observed no changes in differentiation state in myotubes treated with SB for 72 h, which could be seen in Figure 1E. Ectopic expression of MKK6BE in myotubes or p38beta in myoblasts did not alter differentiation states of myotubes (see the figures below).
Figure. Myotube differentiation state is not altered by the expression of MKK6bE, p38β MAPK or the transfection of C/EBP -specific siRNA. Transduction of MKK6bE-encoding adenovirus in C2C12 myotubes (B) and transfection of constitutively active p38 -encoding plasmid (C) and C/EBP -specific siRNA (D) in C2C12 myoblasts were performed as described in Materials and Methods. Myotubes were immunofluorescence-stained with MHC antibody (MF-20) and compared with control myotubes (A). Bar = 100 m.

Q: Response to Q#9: As explained above, the authors' response with regard to excluding other isoforms based on sequence is not valid. My earlier comments were motivated by the apparent activation of pA by kinases in the absence of LAP. This seems not to be the case after addition of statistical details.

A: Okay.

Q: Response to Q#10: The authors respond that C/EBPbeta-silencing does not affect myoblast differentiation. How did they arrive at this conclusion? Evidence should be included. If panel 4A shows extracts from the same cells used in 5C, this should be stated explicitly. Otherwise, the C/EBPbeta control needs to be shown in 5C as it is customary for transient knockdown experiments.

A: Panel D of the above figure demonstrates that C/EBPbeta-silencing did not change myoblast differentiation notably. Here 5C we think meant to be 4C. Yes, this is the case and it is stated explicitly now in figure legend.

Q: Response to Q#15: The authors should include the possibility of host effects in their Discussion.

A: Done (page 15).

© European Molecular Biology Organization

Q: Additional comments:

1) Figure S2: Expression of C/EBPbeta appears enhanced in the last two lanes when assessed by the C/EBPbeta antibody, but not by anti- Flag. How do the authors explain this discrepancy?
2) In March, the following paper came on-line:

C/EBPβ regulates dexamethasone-induced muscle cell atrophy and expression of atrogin-1 and MuRF1.
Gonnella P, Alamdari N, Tizio S, Aversa Z, Petkova V, Hasselgren PO.
J Cell Biochem. 2011 Jul;112(7):1737-48. doi: 10.1002/jcb.23093.
PMID: 21381078

It is appropriate to cite this paper in a relevant context.

A: 1) It might be due to the imperfection of our Western blot technique. As a control experiment for verifying the expression of LAP, the level of Flag is a more direct indication. We did not try to find the exact reason for the difference.
2) Done (page 18).

Acceptance letter 22 July 2011

Many thanks for submitting the revised version of your manuscript. I have now had the chance to look through it, and I am satisfied with your responses to the referees' comments. Thanks also for sending the new SI and text files - we will upload these in place of the previous versions.

I am pleased to be able to tell you that we can now accept your manuscript for publication in EMBOJ - congratulations on a fine piece of work!

Best wishes,
Editor

The EMBO Journal

© European Molecular Biology Organization