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Ubiquitin dependent regulation of MEKK2/3-MEK5-ERK5 signaling module by XIAP and cIAP1

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(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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 20 January 2014

Thank you very much for submitting your study on the regulation of MEKK2/3-MEK5-ERK5 signaling by XIAP for consideration to The EMBO Journal editorial office.

I enclose the comments of two referees that commented on the more general significance and conclusiveness of your work.

While appreciating that your results provide novel insights into potential negative regulation of MEKK/ERK signaling by XIAP-dependent ubiquitination, particularly referee #1 remains at least at this stage unconvinced that this is fully supported by the submitted experimental detail. Though not demanding knockout studies to validate physiological significance, major experimental expansions are demanded to reach a level of convincing molecular clarity.

I recognize that ref#2 appears already more encouraging, though I do have to balance this against the severe concerns raised by ref#1.

Conditioned on such crucial amendments, I am prepared to offer you the opportunity to expand and amend the current dataset for a subsequent peer-review assessment at The EMBO Journal (that will

have to involve the currently very critical ref#1 before being able to reach a final decision).

Please consider your options already at this point rather carefully as to avoid disappointments much later in the process. Please notice that the sole aim of this is to enable rapid proceedings for your results (possibly faster in a less demanding alternative title, though this should be your decision at this stage) and to ensure clear and transparent communication of our rather high demands re further consideration at The EMBO Journal.

Please do not hesitate to get in touch (due to time constrains preferably via e-mail) in case further questions arise OR to discuss anticipated timeline/feasibility of certain experiments OR indeed in case you decide to publish more rapidly elsewhere.

I am sorry to be unable to communicate more enthusiastic news and remain with best regards.

REFEREE REPORTS:

Referee #1:

Takeda et al investigate the regulation of MEKK2/3-MEK5-ERK5 signaling by XIAP. The authors claim that XIAP conjugate K63-linked ubiquitin chains onto MEKK2 and MEKK3, which directly impedes MEK5-ERK5 interaction. As a consequence, XIAP prevents ERK5 activation and loss of XIAP leads to ERK5 hyperactivation and differentiation of primary skeletal muscle cells to myocytes.
The authors claim in their title and abstract that described phenomena apply to IAPs or at least to XIAP and cIAP1. However, they almost exclusively show the data for XIAP. There is no evidence that cIAP1 or cIAP2 can ubiquitylate MEKK2/3. In addition, on page 14 the authors state that cIAP1 loss did not produce the same effect as XIAP loss. Thus, the authors should carefully explore the roles of cIAP1 and cIAP2 in various assays presented in figures 1-7 rather than make general conclusions that are not supported by experimental data.
The knockdown of XIAP and the effect on ERK5 phosphorylation (pERK5) are not very consistent. For example, in figure 1A with better knockdown of XIAP in lane 2 there is smaller increase in pERK5, but less efficient knockdown of XIAP in lane 5 results in much stronger increase in pERK5. Why? And just like in figure 1A, lane 5, in figure 1D there is increase in total ERK5 levels in XIAP -/- cells. Does XIAP affect mRNA or protein levels of ERK5? This needs to be examined.
Interaction between XIAP and MEKK2/3 needs to be further examined. First of all, it is strange that XIAP does not pull down MEKK2 in figure 2B. Second, is this interaction constitutive and does EGF, FGF or FCS stimulation affect it? Third, how can almost all domains of XIAP affect MEKK2 binding (even delta RING has decreased association)? This suggests nonspecific binding. The authors should check individual BIR1, BIR2, BIR3, UBA and RING domains to determine which region of XIAP binds MEKK2 and MEKK3. In figures 2E and 2F the effect of XIAP on MEKK5-MEKK2 is minimal (1.5 fold) prompting a question how can such a small effect on binding create a major effect on ERK5 phosphorylation and myoblast differentiation. In any case, the authors should show XIAP levels in figure 2E so the readers can evaluate increased levels of XIAP, and show if any XIAP is immunoprecipitated in figure 5F. Also, what is shown in 2E is not MEKK2 expression (implying transcription-translation) but the levels of precipitated MEKK2.
In figures 3B, S3A, S3B the authors should show the levels of XIAP and cIAP1 and check if they are auto-ubiquitylated. Just because MEKK2 and MEKK3 are not degraded following FGF treatment does not mean this will necessary be K63-linked ubiquitylation. What about linear ubiquitin chains, or even other chains such as K6, K11 or K48? Related to ubiquitin ligase activity of XIAP, how come there is such a drastic effect of XIAP in kinase assays presented in figure 4, when the absence of XIAP has 1.5 fold effect (top paragraph, page 9) on MEKK2-MEKK5 interaction (figure 2F)? The authors should incubate MEKK2 and MEKK3 in experiments presented in figure 4C and 4D with XIAP only and indicate how much XIAP was used and show XIAP levels by western.
The data in figure 5F is of substandard quality - this experiment should be repeated to generate data that will allow the readers to evaluate presented data. More importantly, the authors should show the formation of endogenous MEKK2-MEKK5-ERK5 complex in wt and XIAP ko or RING mut MEFs with and without stimulation with FGF or EGF.
The data in figure S4D is also hard to interpret - knockdown of MEKK2 or XIAP both stimulate

pERK5 in the background of MEKK2 overexpression. Since overexpressed MEKK2 has huge levels compared to endogenous MEKK2 why and how is MEKK2 knockdown promoting ERK5 phosphorylation?

Referee #2:

ERK5 is a less characterized member of the MAPK family and is activated by various stress stimuli and mitogens. ERK5 is exclusively activated by an upstream MAPKK, MEK5, which in turn can be activated by upstream MAPKKKs such as MEKK2 and MEKK3. It has not been fully understood how the activation of the MEKK2/3-MEK5-ERK5 cascade is negatively regulated. In this study, the authors discovered a ubiquitination-dependent mechanism that suppresses this cascade. They first found that siRNA-mediated knockdown or gene-targeted knockout of the E3 ubiquitin ligase XIAP or cIAP1 leads to ERK5 overactivation in cultured cells (Figs. 1 and S1). XIAP was able to bind to MEKK2/3 and inhibit the MEKK2/3-MEK5 binding in vitro and in cultured cells (Figs. 2 and S2). XIAP and cIAP1 enhanced K63 ubiquitination of MEKK2/3 in vitro and in cultured cells (Figs. 3, S3 and S4). In vitro experiments using purified proteins indicated that K63 ubiquitination of MEKK2/3 does not affect the MEKK2/3-MEK5 binding and the kinase activity of MEKK2/3 toward MEK5, but it inhibits the MEK5-ERK5 binding and MEKK2/3-MEK5-driven ERK5 activation (Figs. 4, 5, S5 and S6). Knockdown of XIAP in cultured myoblasts enhanced myogenesis in a manner dependent on overactivation of the MEKK2/3-MEK5-ERK5 cascade (Figs. 6, 7 and S7). Thus, XIAP-mediated negative regulation of the MEKK2/3-MEK5-ERK5 cascade is biologically relevant in cells.

The experimental results presented in this study are convincing enough to support the authors' conclusion and will provide novel insights into the regulatory mechanism of the MEKK2/3-MEK5-ERK5 cascade. Therefore, I recommend publication of this study if following minor points are properly addressed.

1) In page 8, lines 8-10, it seems to me that the authors explain their experimental results. However, no data is shown.

2) In page 10, lines 18-20, "stable expression of XIAP-H467A but not wild type XIAP rescued FGF-induced ERK5 activation in the XIAP deficient MEFs" may be "stable expression of wild type XIAP, but not XIAP-H467A, rescued FGF-induced ERK5 overactivation in the XIAP deficient MEFs".

3) In the legend of Fig. 3C, "XIAP-H467" would be "XIAP-H467A".

4) In Fig. 5B, "GST+MEKK2(Ubi)n" and "GST+MEKK2-NU" would be "GST-MEKK2(Ubi)n" and "GST-MEKK2-NU", respectively.

5) In the legend of Fig. 5B, "MEKK2 was immunoprecipitated" would be "MEKK2 was pulled down".

6) In Fig. 5C, MEKK2 conjugated with His-ubiquitin was pulled down by Ni-NTA (right lane). I cannot understand why non-ubiquitinated (non-His tagged) MEKK2 could also be pulled down by Ni-NTA (left lane).

7) The authors should not publish the result from a one-time experiment (Fig. 7C, left and middle graphs). They should repeat experiments or eliminate these graphs.

8) In Fig. S3C, the authors should indicate (like Fig. 3C) that a control IgG was used for immunoprecipitation in the leftmost lane.

1st Revision - authors' response 28 January 2014

We thank the reviewers for their comments and constructive criticisms and please find our point-by-point response below.

Referee #1:

Takeda et al investigate the regulation of MEKK2/3-MEK5-ERK5 signaling by XIAP. The authors claim that XIAP conjugate K63-linked ubiquitin chains onto MEKK2 and MEKK3, which directly impedes MEK5-ERK5 interaction. As a consequence, XIAP prevents ERK5 activation and loss of XIAP leads to ERK5 hyperactivation and differentiation of primary skeletal muscle cells to myocytes.The authors claim in their title and abstract that described phenomena apply to IAPs or at least to XIAP and cIAP1. However, they almost exclusively show the data for XIAP. There is no evidence that cIAP1 or cIAP2 can ubiquitylate MEKK2/3. In addition, on page 14 the authors state that cIAP1 loss did not produce the same effect as XIAP loss. Thus, the authors should carefully explore the roles of cIAP1 and cIAP2 in various assays presented in figures 1-7 rather than make general conclusions that are not supported by experimental data.

We thank the reviewer for his/her evaluation. We have made substantial efforts to address all the concerns of this reviewer as detailed in our responses below. We hope that the reviewer is now convinced to allow us to publish these observations.

As suggested by the reviewer, we first checked if cIAP2 depletion with two different sets of validated siRNAs led to any alterations in pERK5 levels. Interestingly, depletion of cIAP2 failed to increase pERK5 levels unlike cIAP1 or XIAP. These data are now added to the manuscript (find the new Figures S1B). As XIAP-cIAP1 complex primarily contributed to ERK5 inactivation, we have decided to change the title of the manuscript to avoid any misinterpretation. The title has now been changed to “Ubiquitin dependent regulation of MEKK2/3-MEK5-ERK5 signaling module by XIAP and cIAP1”.

We would like to however indicate that we have indeed shown that cIAP1 can directly ubiquitinate MEKK2/3 both in vitro and in vivo and these data were presented in the supplement (Please see figures S3A-S3C). Further, the fact that loss of cIAP1 did not mimic XIAP depletion in human myogenic

differentiation is possibly due to the cross regulation of IAPs. We detect a strong stabilization of XIAP protein upon cIAP-1 depletion in human myocytes.

Please find these data presented below. We are developing a manuscript describing the cross regulation between these IAPs and further analyses on how their RING activity is regulated in a heteromeric complex. We will be glad to add these data to the Supplementary data if advised by the reviewer.

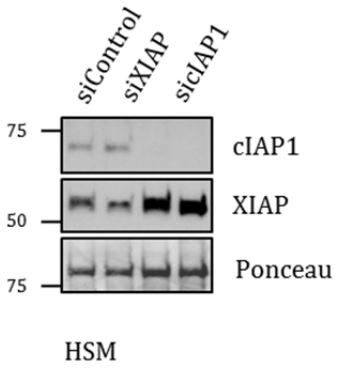
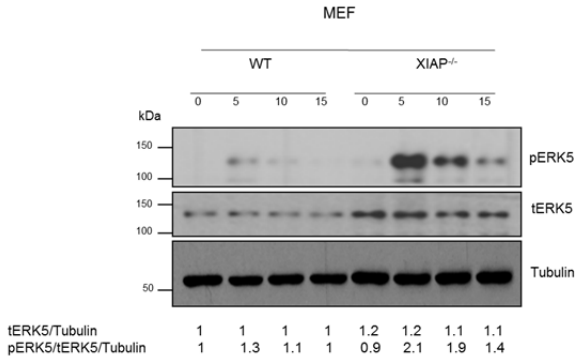


Figure legend: Human primary skeletal myoblasts are transfected with control or siRNAs directed against various IAPs. The knock down efficiency was monitored by immunoblots. Staining of the entire membrane with ponceau was employed as a loading control.

The knockdown of XIAP and the effect on ERK5 phosphorylation (pERK5) are not very consistent. For example, in figure 1A with better knockdown of XIAP in lane 2 there is smaller increase in pERK5, but less efficient knockdown of XIAP in lane 5 results in much stronger increase in pERK5. Why? And just like in figure 1A, lane 5, in figure 1D there is increase in total ERK5 levels in XIAP -/- cells. Does XIAP affect mRNA or protein levels of ERK5? This needs to be examined.

In response to the reviewer's suggestion we have repeated these experiments with all the four sets of siRNAs with new hands (another author of this manuscript) and these data are fully reproducible. In addition, we have presented data from other cell lines, primary human cells, myocytes and MEFs derived from XIAP deficient mice all demonstrating that loss of XIAP indeed led to an increase in pERK5 levels. As a final confirmation, we have also performed complementation assays to rule out any potential off-target effects (Fig. S1E). We have also carefully validated the specificity of the pERK5 antibody by employing ERK5 siRNAs (Fig. 1B). The variation in pERK5 levels in some blots



could possibly be due to developing and loading issues. We have presented the new data as Figure 1A. Again, we don't detect any significant increase in total ERK5 levels. The data presented in 1D has been quantified and these analyses revealed that loss of XIAP did not lead to any significant increase in total erk5 levels. Please find these analyses presented for the eyes of the reviewer.

Interaction between XIAP and MEKK2/3 needs to be further examined. First of all, it is strange that XIAP does not pull down MEKK2 in figure 2B. Second, is this interaction constitutive and does EGF, FGF or FCS stimulation affect it? Third, how can almost all domains of XIAP affect MEKK2 binding (even delta RING has decreased association)? This suggests nonspecific binding. The authors should check individual BIR1, BIR2, BIR3, UBA and RING domains to determine which region of XIAP binds MEKK2 and MEKK3.

We thank the reviewer for this suggestion. The fact that endogenous MEKK2 does not co-precipitate with XIAP could possibly be due to the antibody targeting the interaction sites or the epitope is not recognized due the proteins interacting in this complex. We have also tried a couple of other antibodies and they fail to detect MEKK2 in cell lysates and thus we are confined to this antibody from Cell signaling. Further, we could not detect any significant increase in the interaction between XIAP and MEKK2 in response to growth factors. However, to further evaluate the domain(s) mediating the interaction, we have generated new constructs and purified individual BIR domains as advised by the reviewer. In addition, unlike the previous attempts, we have also slightly modified the protocol as there was some background binding of MEKK2 to the beads, which has prompted us to employ some high stringency washing conditions.

We have employed a blocking step with BSA and conducted all our washes after

the precipitation with buffer containing 150 mM NaCl. Under these settings, with the newly purified proteins, we could demonstrate that BIR1 and BIR2 domains possibly mediate the interaction between XIAP and MEKK2. Please find our new figures S2A and S2B. Nevertheless, BIR1 or BIR2 domains bind with less affinity when compared to full length XIAP. The materials and methods section and the main text have all been updated with these new observations.

In figures 2E and 2F the effect of XIAP on MEKK5-MEKK2 is minimal (1.5 fold) prompting a question how can such a small effect on binding create a major effect on ERK5 phosphorylation and myoblast differentiation. In any case, the authors should show XIAP levels in figure 2E so the readers can evaluate increased levels of XIAP, and show if any XIAP is immunoprecipitated in figure 5F.Also, what is shown in 2E is not MEKK2 expression (implying transcription-translation) but the levels of precipitated MEKK2.

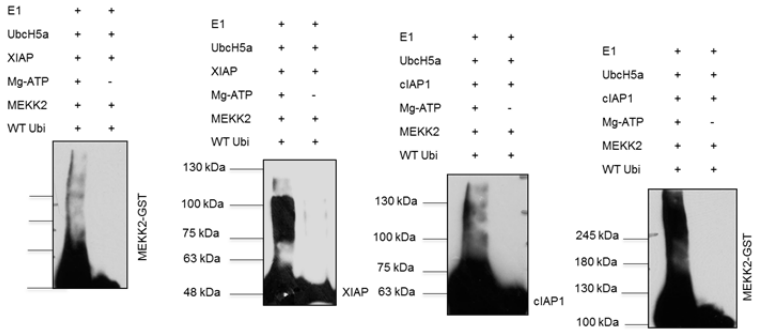
We apologize that we didn't make things much clearer. As XIAP directly binds to MEKK2 and MEKK3 in a PB1 domain dependent manner (Figure 2C), it was tempting to test if the competition is direct and if there is an increased interaction between MEKK2 and MEK5 in the absence of XIAP. Regarding 2E, the data presented are from an in vitro reconstitution experiment (with the quantification from three independent experiments) to evaluate a direct role of XIAP in competing with the PB1-mediated interaction between MEK5 and MEKK2 by employing purified proteins. Further, we also detect that XIAP fail to bind to MEK5 (Figure 2D). Thus most of the XIAP employed to compete with MEKK2 is in the supernatant which is usually discarded. We have repeated these experiments again and preserved the supernatant to detect XIAP levels. These data are now presented as main figure 2E. It is evident that we have indeed employed increasing concentrations of XIAP in the reaction mix.

Consistent with these observations, there is indeed an increase in the interaction between MEK5 and MEKK2 in vivo in the absence of XIAP at steady state levels and after stimulation with growth factors (Figure 2F and S2C). Nevertheless, we have detected XIAP-dependent ubiquitination of MEKK2 in response to growth factors (Figure 3A) and further both XIAP and cIAP1 can directly ubiquitinate MEKK2/3. Interestingly, the phenomenon of XIAP regulating ERK5 is clearly

RING domain-ubiquitin dependent (Figure 3F and 3G) as loss of the RING activity or RING domain failed to rescue ERK5 inactivation. These results have only prompted us to investigate the direct role of ubiquitination in the activation dynamics of this cascade. Thus XIAP-cIAP1 complex regulates ERK5 inactivation is dependent on ubiquitination of MEKK2/3. Please see our response regarding figure 5F below.

In figures 3B, S3A, S3B the authors should show the levels of XIAP and cIAP1 and check if they are auto-ubiquitylated.

We have consistently detected autoubiquitination of XIAP or cIAP-1 in addition to substrate ubiquitination in these assays. We have repeated these experiments and consistently, we detect autoubiquitination of these two IAPs in the reaction mix. If requested, we can also add these data to the supplement. We have nevertheless indicated the same in the main text.



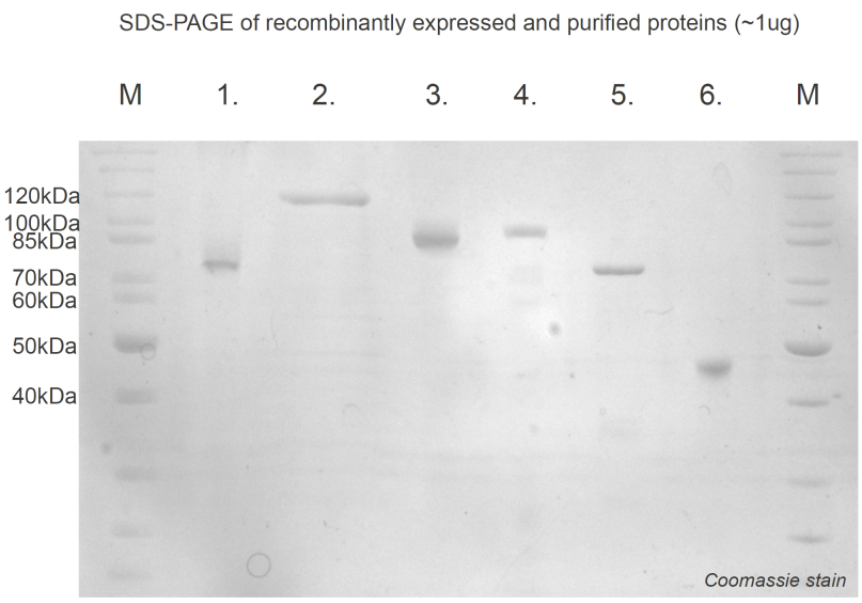
Just because MEKK2 and MEKK3 are not degraded following FGF treatment does not mean this will necessary be K63-linked ubiquitylation. What about linear ubiquitin chains, or even other chains such as K6, K11 or K48?

Yes indeed! We agree and very much share the concern of reviewer on this issue. However, we are very much limited by the tools currently available to precisely decipher the kind of ubiquitin chains. We re-assessed the mass spec data and we detected traces of K-63, K-48, K29 and K11 ubiquitin in the screen which has primed us to screen further with these ubiquitin mutants (though they carry 6 lysine mutations) as well as with the antibodies developed by Vishva

Dixit lab. All these experiments indicate that IAPs predominantly synthesize K-63 chains on MEKK2 and MEKK3 both in vitro and in vivo. In addition, we now performed experiments with deubiquitinase (DUB) AMSH (AMSH [associated molecule with the Src homology 3 domain of signal transducing adaptor molecule (STAM)] a K-63 chain-specific DUB. As expected, treatment with AMSH cleaves of ubiquitin from MEKK2 (ubiquitinated with XIAP) similar to the broad spectrum DUB, Usp2. These results further support the notion that the chains synthesized on MEKK2 are predominantly K-63 chains. These data are now added to Supplement (Please see new figure S3F). Nevertheless, we don't want to rule out the possibility of other kind of chains or even mixed chains. We have now changed the text (as "predominantly K-63 linked chains") throughout including the abstract accordingly. However, we have characterized the functional-biological significance of this ubiquitination. Ubiquitination of MEKK2 or MEKK3 by IAPs did not influence their kinase activity or their direct interaction with MEK5. However, ubiquitinated MEKK2/3 directly impedes the complex formation between MEK5 and ERK5 in a trimeric complex.

Related to ubiquitin ligase activity of XIAP, how come there is such a drastic effect of XIAP in kinase `assays presented in figure 4, when the absence of XIAP has 1.5 fold effect (top paragraph, page 9) on MEKK2-MEKK5 interaction (figure 2F)? The authors should incubate MEKK2 and MEKK3 in experiments presented in figure 4C and 4D with XIAP only and indicate how much XIAP was used and show XIAP levels by western.

We would like to reiterate that regulation of ERK5 activation by IAPs is RING/ubiquitination dependent. The experiments presented in Figure 4C and 4D and Figure S5 constitute coupled ubiquitination-phosphorylation assays by employing purified components. Here, we have consistently employed (MEKK2/3: 0.2µM, MEK5: 1µM and ERK5: 5µM). This is now mentioned in the legends. And we have checked the quality and the concentrations of all the proteins employed in these experiments.



1. GST-XIAP
2. MBP-MEKK3
3. MBP-MKK5
4. MBP-ERK5 Kinase domain
5. GST-MKK7
6. JNK1

And the reaction mix was pipetted out for various time points to measure the kinase activity. Thus the concentration of XIAP remains the same in all the time points. Nevertheless, we have also tested if the indicated concentration of XIAP can inhibit ERK5 phosphorylation in the absence of E1 and E2 (in the absence of ubiquitination). As expected, XIAP at these indicated concentrations failed to prevent ERK5 phosphorylation. These data are now added in supplement (as Figure S5A). In addition, we also would like to indicate that we have conducted experiment with JNK as a control (presented in the supplement as S5B) and clearly, ubiquitination of MEKK2 or MEKK3 failed to prevent JNK phosphorylation confirming the SPECIFIC role of ubiquitination in the inactivation of ERK5 but not JNK pathway. Thus the direct effect detected on ERK5 phosphorylation is clearly dependent on the ubiquitination of MEKK2 (also consistent with Figs 3F and 3G). These results indeed reveal a novel role for ubiquitin in the direct inactivation of the ERK5 cascade.

The data in figure 5F is of substandard quality - this experiment should be repeated to generate data that will allow the readers to evaluate presented data. More importantly, the authors should show the formation of endogenous MEKK2-MEKK5-ERK5 complex in wt and XIAP ko or RING mut MEFs with and without stimulation with FGF or EGF.

In figure 5F, we have tested the influence of ubiquitination (on MEKK2) on the formation of trimeric complex by employing purified components to detect any direct role. After the ubiquitination reaction, the components were mixed and MEK5 was precipitated with MBP pull down and the co-precipitated MEKK2 and ERK5 was checked. As XIAP do not bind to MEK5 we could not detect any XIAP in complex with MEK5. The amount of ERK5 co-precipitated with MEK5 was quantified and we could detect a decrease when MEKK2 is ubiquitinated (Please see S6A with the quantifications). These data are now moved to the supplement and presented as Figure S6A. We have also performed identical experiment and employed a MEK5 antibody to immunoprecipitate MEK-5 and reproduced the data with the same results. These data are now presented as main figures (Figure 5F) as similar amounts of MEK5 were immunoprecipitated in both conditions.

As suggested by the reviewer, we have made several attempts to precipitate the endogenous MEKK2/3-MEK5-ERK5 complex. To our knowledge, all the attempts to purify the entire MAPK module (MAPKKK-MAPKK-MAPK) under steady state and stimulated conditions at endogenous levels have not been successful even for other MAPK modules due to the transient nature of these complexes. As far as ERK5 is concerned, there could also be spatial separation as there are reports that MEKK2 and MEK5 can shuttle between the nucleus and cytosol (Raviv et al J Cell Sci. 2004 Apr 1;117(Pt 9):1773-84). While we are successful in precipitating MEK5 and MEKK2 (presented as figures 2F and S2C) we fail to detect ERK5 in these experiments. We have also tried the reverse IPs, and the antibodies against ERK5 are not efficient in immunoprecipitating total ERK5 from cells.

As suggested by the editors, we have employed cross linking agents like DSS. After the quenching period, we were no longer able to immunoprecipitate MEKK2

from these cells. As all these attempts failed, we have decided to establish a stable cell line expressing tagged version of MEK5 and ERK5. To this end, we have generated a HeLa cell line with Flag tagged ERK5 and V5-tagged MEK5. First loss of XIAP led to an increase in pERK5 levels. As expected, when ERK5 is pulled down with FLAG-beads, we could detect increased amounts of MEK5 with ERK5 in the absence of XIAP thus confirming our hypothesis.

The data in figure S4D is also hard to interpret - knockdown of MEKK2 or XIAP both stimulate pERK5 in the background of MEKK2 overexpression. Since overexpressed MEKK2 has huge levels compared to endogenous MEKK2 why and how is MEKK2 knockdown promoting ERK5 phosphorylation?

The aim of this experiment was to accomplish a complementation approach by employing a 3'UTR siRNA. It was known from the work of Gary Johnson that mere overexpression of MEKK2 will increase pERK5 levels. We aim to compare the pERK5 levels in cells stably expressing wild type as well as the MEKK2K450R mutants after depleting endogenous MEKK2. Indeed, expression of K450R mutant led to relatively higher pERK5 levels when compared to the wild type expressing cells. The rationale behind XIAP knock down was to check if there is any additive increase in pERK5 levels. However, we agree with the reviewer that the manner of presentation here would make things hard to interpret and thus we have decided to remove this data from the manuscript. Our initial screen suggests that lysine 450 might be the key residue here but substantial experiments are clearly required to prove this point. We hope to clarify these issues in the follow up story soon.

Referee #2:

ERK5 is a less characterized member of the MAPK family and is activated by various stress stimuli and mitogens. ERK5 is exclusively activated by an upstream MAPKK, MEK5, which in turn can be activated by upstream MAPKKKs such as MEKK2 and MEKK3. It has

not been fully understood how the activation of the MEKK2/3-MEK5-ERK5 cascade is negatively regulated. In this study, the authors discovered a ubiquitination-dependent mechanism that suppresses this cascade. They first found that siRNA-mediated knockdown or gene-targeted knockout of the E3 ubiquitin ligase XIAP or cIAP1 leads to ERK5 overactivation in cultured cells (Figs. 1 and S1). XIAP was able to bind to MEKK2/3 and inhibit the MEKK2/3-MEK5 binding in vitro and in cultured cells (Figs. 2 and S2). XIAP and cIAP1 enhanced K63 ubiquitination of MEKK2/3 in vitro and in cultured cells (Figs. 3, S3 and S4). In vitro experiments using purified proteins indicated that K63 ubiquitination of MEKK2/3 does not affect the MEKK2/3-MEK5
binding and the kinase activity of MEKK2/3 toward MEK5, but it inhibits the MEK5-ERK5 binding and MEKK2/3-MEK5-driven ERK5 activation (Figs. 4, 5, S5 and S6). Knockdown of XIAP in cultured myoblasts enhanced myogenesis in a manner dependent on overactivation of the MEKK2/3-MEK5-ERK5 cascade (Figs. 6, 7 and S7). Thus, XIAP-mediated negative regulation of the MEKK2/3-MEK5-ERK5 cascade is biologically relevant in cells.The experimental results presented in this study are convincing enough to support the authors' conclusion and will provide novel insights into the regulatory mechanism of the MEKK2/3-MEK5-ERK5 cascade. Therefore, I recommend publication of this study if following minor points are properly addressed.

We sincerely thank the reviewer for appreciating the importance of the study and for his/her support for publishing these observations. We have duly addressed the suggestions of the reviewer and made the requisite changes as listed below.

1) In page 8, lines 8-10, it seems to me that the authors explain their experimental results. However, no data is shown.

This mistake has been corrected. This sentence refers to Figures 1E and 1F.

2) *In page 10, lines 18-20, "stable expression of XIAP-H467A but not wild type XIAP rescued FGF-induced ERK5 activation in the XIAP deficient MEFs" may be "stable expression of wild type XIAP, but not XIAP-H467A, rescued FGF-induced ERK5 overactivation in the XIAP deficient MEFs".*

This sentence has been changed as suggested.

3) *In the legend of Fig. 3C, "XIAP-H467" would be "XIAP-H467A".*

Thanks for pointing out this mistake. This has been corrected.

4) *In Fig. 5B, "GST+MEKK2(Ubi)n" and "GST+MEKK2-NU" would be "GST-MEKK2(Ubi)n" and "GST-MEKK2-NU", respectively.*

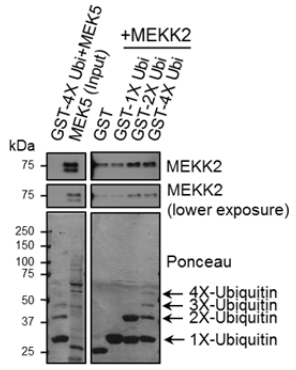
This has been corrected now.

5) In the legend of Fig. 5B, "MEKK2 was immunoprecipitated" would be "MEKK2 was pulled down".

This has been corrected now.

6) In Fig. 5C, MEKK2 conjugated with His-ubiquitin was pulled down by Ni-NTA (right lane). I cannot understand why non-ubiquitinated (non-His tagged) MEKK2 could also be pulled down by Ni-NTA (left lane).

That's indeed a good point. In this reaction there is free ubiquitin and we subsequently identified that MEKK2 is an ubiquitin binding protein. Please find these data below. These observations call for potential ubiquitin binding domains and the role of such domains in regulating activity of this kinase. We hope to investigate these issues as soon as we publish these observations.



7) *The authors should not publish the result from a one-time experiment (Fig. 7C, left and middle graphs). They should repeat experiments or eliminate these graphs.*

Fully Agreed! We have exchanged these figures as suggested. However, these are experiments performed with primary cell lines in their early passages and are fully reproduced. Thus we have left these data in the supplement. If the reviewer insists we will be glad to remove it all together from the manuscript.

8*) In Fig. S3C, the authors should indicate (like Fig. 3C) that a control IgG was used for immunoprecipitation in the leftmost lane.*

Thanks for pointing this out. We have included it.

2nd Editorial Decision 13 May 2014

Please find the comments on your revised study from the critical referee attached below. As you will see, there are a few remaining items that would need your attention/re-arranging the article before ultimate acceptance for publication here.
I would be delighted to receive an ultimate version, including necessary source data files (you may remember: one PDF per relevant figure) to your earliest convenience.

Further, it would be most helpful if you were to provide a short, (min two - up to four bullet-point) summary to outline major advances provided by your study.

I trust that you will meticulously integrate the requested final amendments and look forward to receiving the finalized files that should enable formal acceptance.

REFEREE REPORT:

Referee #1:

The authors have attempted to address the issues raised by reviews by providing explanations for their results and by performing several additional experiments. The manuscript has been improved but there are still few remaining issues that should be resolved.
- In figure 1B the authors should include tubulin western blot as a loading control since it shows there is overall higher level of protein expression in XIAP -/- MEFs thus explaining elevated ERK5 levels. Ponceau S staining can be removed.
- The authors should show the data that growth factors do not affect XIAP-MEKK2 interaction as supplemental figure and discuss this fact in the text.
- The lack of effect of cIAP1 knockdown makes me believe this is XIAP-selective process and it would be better to characterize it this way (but still include cIAP1/2 data for the complete disclosure).
- In figures 3 and S3 with ub assay the authors should show the western blots of XIAP and cIAP1 levels and autoubiquitination.

