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Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF- κ B regulation

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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.)

1st Editorial Decision

26 April 2012

Thank you again for submitting your manuscript for consideration by The EMBO Journal. Three expert referees have now reviewed it, and provided their comments as copied below. While the referees consider your findings and conclusions interesting in principle, they at the same time all raise a number of important concerns that would need to be decisively addressed before eventual publication.

Given the overall interest of the work, I would like to give you an opportunity to revise the manuscript in response to the referee's comments. Since we allow only a single round of major revision, it will be important that you adequately respond to all the points raised at this stage in the process. However, there are a few issues that will be particularly important:

- the physiological significance of several experiments needs to be strengthened as suggested by the referees, e.g. by use of TNF stimulation, A20-deficient cells, analysis of endogenous protein interactions
- all three referees (ref 1 pt 7, ref 2 pt 3, ref 3) request some further understanding of the actual mechanism of inhibition mediated by ZF7-linear ubiquitin binding
- referee 3 indicates several concerns with the interpretation and presentation of the structural analyses, which should be addressed
- from an editorial point of view: please reduce the number of supplementary figures by incorporating more relevant data into the main manuscript (which currently only has 4 figures!), making use of the full-length format of an EMBO Journal article. For example, it might be good to move structural comparisons as shown in SI Fig. 9 into the main manuscript, given the current interest in ubiquitin chain recognition modes
- several figure panels showing gels or blots (e.g. Fig 2A/B, Fig 4B/C, SI Fig 4C, SI Fig 11) appear to have too much contrast and show almost no background signals anymore. Please adjust this to

allow a better representation of the original data - as is the case for example in the blots in SI Fig 1A/C.

- please include PDB accession codes of the newly determined structures in your revised manuscript
- finally, I should remind you that The EMBO Journal encourages accurate and complete citation of the most relevant primary literature; we allow unlimited references to be cited in order to facilitate this. I would greatly appreciate if you reviewed your citations and added relevant citations that were missed originally (e.g. p16 Sato et al EMBOJ 28:2461 for RAP80-UIM-K63 chain structures; p16 Kulathu et al NSMB 2009 for TAB2-NZF-K63 binding in addition to Sato et al 2009)

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

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication - I look forward to your revision. Should you have any further questions regarding this decision or the reports, please do not hesitate to contact me.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1

In this manuscript, Tokunaga et al. investigate the regulation of NF- κ B by LUBAC and A20. Although CYLD and A20 both inhibit LUBAC-induced NF- κ B, the mechanisms appear to be distinct. CYLD requires its DUB activity to suppress NF- κ B by LUBAC, whereas A20 requires its seventh zinc finger (ZF7). A20 ZF7 suppresses NF- κ B by interacting with linear polyubiquitin chains. The authors used X-ray crystallography to determine the precise binding sites of A20 ZF7 and diubiquitin and the molecular nature of the interactions. Interestingly, genetic alterations in the ubiquitin binding sites in ZF7 have been observed in B-cell lymphomas suggesting that loss of linear ubiquitin binding by A20 may contribute to lymphomagenesis. A20 ZF7 specifically interacts with the HOIP subunit of LUBAC and recruits A20 to the LUBAC-IKK complex. The findings in this report on A20 and the negative regulation of LUBAC and linear ubiquitination are interesting and important. However, as noted below there are concerns with the functional studies which are largely based on overexpression strategies in 293-T cells. Also, the authors have failed to demonstrate the precise mechanism by which A20 inhibits LUBAC and NF- κ B after it is recruited to LUBAC-IKK.

1) It has been controversial whether A20 requires its DUB activity to inhibit NF- κ B. Overexpression of A20 mutants in 293-T cells may yield misleading results since these cells have high basal levels of A20 which may interact with the overexpressed forms of A20 and influence NF- κ B activation. Therefore, some of the key results in Fig. 1 need to be repeated in an A20-null (either knockout or siRNA knockdown) background. The authors also need to show expression of transfected proteins (i.e. Figs. 1B-F, 3B).

3) Overexpression of LUBAC (Fig. 1B, 4A) and TRAF6 (Fig. 1E) is highly artificial. The authors should also repeat these experiments with stimulation (i.e. TNF or IL-1).

4) Fig. 4A lacks the wild-type A20 control. Supp. Fig. 10 lacks controls with the A20 mutants alone. Supp. Fig. 10 should also have positive controls for ABIN-1 and TAX1BP1 (i.e. inhibition of IL-1-induced NF- κ B).

5) Generally, more experiments are needed with endogenous proteins in the manuscript to confirm overexpression results (i.e. co-IPs for endogenous A20, HOIP, HOIL-1L, Sharpin and NEMO binding). Stimulation of cells with TNF for various time-points will reveal if these interactions are stimulus-dependent.

6) In the absence of A20 (either knockdown or genetic deficiency), is linear ubiquitination of NEMO enhanced and more persistent after TNF stimulation? The authors should examine endogenous linear ubiquitination of NEMO using the linkage-specific linear Ub antibody previously used by the authors (Tokunaga et al 2009 Nat. Cell Biol. 11: 123-32).

7) As mentioned above, a weakness of the paper is that the mechanism of A20 inhibition of LUBAC-induced NF- κ B activation remains unknown. Although ZF7 binds to linear Ub chains and recruits A20 to LUBAC-IKK, how does A20 actually inhibit NF- κ B? The authors should at least discuss potential models.

Referee #2

In the manuscript 'Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF- κ B regulation', Tokunaga, Nishimatsu and colleagues have shown a negative regulation of LUBAC-induced NF- κ B signaling by A20-ZF7 that is independent from its DUB activity. Tokunaga et al. have for the first time shown that A20 and linear polyubiquitin chains interact via A20-ZF7 and solved the co-crystal structure of A20-ZF7 and di-linear ubiquitin chains. These findings have a high potential to attract the readers of EMBO Journal. However, before the publication, some critical points need to be addressed.

Major points:

1. In general: from the data provided in the manuscript, it is clear that A20 inhibits LUBAC-mediated NF- κ B activation by binding to linear ubiquitin chains in the system they used. The authors examined the effects of different A20 mutants on LUBAC-induced NF- κ B activation by using NF- κ B reporter assay in HEK293T cells overexpressing A20 wt or mutants (Fig 1 A-E, Fig 3D, Fig 4A, Supp Fig 4A, Supp Fig 10 A and B). The major concern is that whether the negative effects observed in the manuscript is just dependent on the overexpression of linear-ubiquitin binding proteins especially by looking at the results of Supp Figure 4A in which authors used tandem repeats of ZF7X2 and ZF7X3. In this figure, single ZF7 alone has almost no effects on LUBAC-induced NF- κ B activation but the effects become drastic when ZF7X2 or ZF7X3 was used in the assay. Even though they showed that ABIN-1 overexpression has little effects on LUBAC-induced NF- κ B activation (Supp Figure 10A), the authors need to show the specific role of A20-ZF7 by comparing with other linear-ubiquitin binding domains, such as NEMO-UBAN X1, X2 or X3 or ABIN-UBANx1, X2 or X3 using the same technique as Supp Fig 4A.
2. In general: the effects of full-length A20 mutants, which do not interact with linear-ubiquitin chains were exclusively tested in an overexpression system. The authors also need to show the role of A20-ZF7 by generating the A20-ZF7 mutants (N772 mutant and E781 mutant) expressing cells in the background of A20-knock out or A20-knock down. These cells then need to be stimulated by TNF- α (and/or other cytokines) and NF- κ B activation needs to be examined.
3. In general, following up the question above (#2): one of the major points unclear in the manuscript is the actual role of A20-ZF7 binding to linear ubiquitin chain. If A20 does not catalyze linear ubiquitin chains, either free chains or the ones on NEMO, what does A20 do by binding to linear ubiquitin chain? The authors showed that A20 gets recruited into the LUBAC-IKK complex however it is still not yet clear how it controls the NF- κ B activation. Does A20 disturb the complex formation of positive regulator of NF- κ B downstream or inhibit IKK activity or any downstream molecules? This point is not clear and needs to be addressed by using the A20 mutant cells (as suggested in #2), analyzing the TNFR complex formation or TAK1-mediated IKK activation as the authors discussed in the text in page 18.
4. Figure 1F: The authors tried to show in this figure that the effect of A20-ZF7 is dependent on LUBAC by using wt and HOIL-1L deficient MEFs. It is clear that ZF1-7 is required for the

inhibitory effects on NF- κ B in wt MEFs but not in HOIL-1 KO MEFs. On the other hand, these data lead to a question if HOIL-1L deficiency in MEFs lead complete loss of linear ubiquitination upon TNF- α stimulation since Sharpin should still plays a role in this condition. If Sharpin also plays a role in linear ubiquitination as the authors previously published (Tokunaga et al, 2011), ZF1-7 in HOIL-1L deficient cells should still inhibit certain extent of NF- κ B activation. The authors need to clarify this point by comparing with Sharpin deficient cells and/or Sharpin/HOIL-1L double deficient cells.

Minor point:

1. In general: Authors need to distinguish clearly in the text if the effects of A20 on the NF- κ B activation is LUBAC mediated or not. In some sentences, it is only described as 'A20 suppressed NF- κ B activation independently to the DUB catalytic activity' but it should be written as 'A20 suppressed LUBAC-induced NF- κ B activation independently to the DUB catalytic activity'. This is critical since A20 was shown to have negative effects on the NF- κ B signaling through RIP1 in a distinct mechanism.

Referee #3

The manuscript by Tokunaga et al describes the structure of ZF7 of the deubiquitinase A20 bound to linear di and tetraubiquitin. Based on the structure and luciferase-based NF- κ B activation assays the authors suggest a model in which A20 suppresses NF- κ B activation through binding to linear ubiquitin chains rather than by its DUB activity as exemplified by CYLD.

This is a very interesting and intriguing model that should appeal to a large audience. However, in its current form the manuscript raises a number of questions that have not been sufficiently addressed and create much confusion. I would have really liked to see the authors push the biology a bit further and dig deeper to figure out how precisely the suppression of NF- κ B works or at least provide a clear model.

I think part of the problem with this manuscript is due to the write-up, which is not always as clear and structured as it could be and at present sounds more like an assembly of multiple small stories and not a finished, coherent story. The link between the structure and cellular data seems a bit over-engineered and the authors should put more effort into explaining why particular experiments have been done (the ABIN-1/TAXBP1 paragraph for example).

Major points

In Figure S4A the authors show that the isolated ZF7 has hardly any effect on NF- κ B activation, which the authors explain by its low expression. However, in Figure 1C the authors see a relatively strong effect upon expression of ZF6-7. Is this really purely due to levels of protein expression? If yes, the authors need to show protein expression levels in all activation assays to support this statement.

What point are the authors trying to make with the paragraph "TRAF6 is an E3 ligase..." What is the difference between TRAF6 and LUBAC mediated NF- κ B activation? Please give the reader a bit more information, are the authors trying to imply that this might be due to different ubiquitin chains being involved? If yes, have the paragraph later and say so explicitly. If the authors think there are other reasons they should say so as well.

Given the low level of input of K48 chains versus K63 chains in Figure 5C I'm not convinced that the residual binding to K48 chains shown is not almost as strong as to K63 chains in comparison. The point I'm trying to make is that A20 ZF7 binding to linear chains is convincing and specific whereas the binding to K63 chains is in my opinion just an unspecific artifact, probably exaggerated by doing pulldown assays and hence doesn't require the amount of discussion it gets on page 17.

The description of the different crystal forms and their packing is rather confusing and I seriously doubt that a non-structural biologist will understand the description of the filaments. The authors need to explain better how the ZF is capable of stitching the diubiquitin together to form these filaments. What type of polyubiquitin chains have been used for the EM pictures - long chains? The authors indicate that it is unlikely that this filament formation plays a role in vivo - if this is the case I think it might be better to remove the EM pictures as this just confuses the story. On the other hand, if they think there might be something to it they have to investigate it in more detail - for

example make mutants that should disrupt filament formation and then test them in NF- κ B assays and test other domains specific for linear chains such as the UBAN of NEMO for their ability to induce filament formation.

Why don't the authors show the structure of the ZF bound to tetraubiquitin? Do 2 ZF bind to tetraubiquitin? Please add this as a supplemental Figure.

How do the authors know that they have potassium bound and what is the role of this ion in this structure? Isn't that highly unusual? This needs to be further explained.

How can Cys767 which coordinates the Zn²⁺ ion form a salt bridge? I think what the authors are referring to is at most a hydrogen bond.

The inset showing the Zn²⁺ and K⁺ coordination is not very clear. This Figure should be redrawn showing much more clearly which residues coordinate what. A schematic might be more useful here than the actual structure.

Similarly, I don't think Figure 3B shows very well the "the recognition of a hydrophobic patch by shape complementarity...."

How have the authors ensured that the mutants that lost their ability to bind ubiquitin are not unfolded? This should be tested by CD spectroscopy or similar.

Why does the N772A mutant which completely lost the ability to bind linear di-Ub still suppresses NF- κ B activation nearly as well as wt? This absolutely doesn't make sense and needs to be explained.

I'm very confused about the last paragraph of Results which shows an interaction between the HOIP subunit of LUBAC and ZF7. How does this fit with the ubiquitin binding property of this ZF? Are the authors suggesting that it can bind both, HOIP and linear ubiquitin chains? I think this raises a fundamental question about the model suggested in this manuscript: do the authors suggest that the linear ubiquitin-binding properties of A20 ZF7 will sequester linear chains away from the UBAN of NEMO or alternatively "block" linear chains attached to NEMO and thereby inhibit activation of NF- κ B or do the authors think that the ZF binds HOIP and thereby somehow interferes with NF- κ B activation. It appears that in the discussion on page 18 the authors try to keep everybody happy by saying that this ZF can do whatever required - I find this rather frustrating and would like the authors to present a more explicit model that is supported by their data.

Have the authors looked at binding of ZF7 to monoUb? Given that it is not the Ub-Ub linkage that is recognized by the ZF, monoUb should bind just with lower affinity. This experiment should be included, ideally by ITC as this would also give the stoichiometry of the interaction

Minor points

The authors say in the abstract and introduction that the "determined the structures of A2- ZF7 in complex with linear diubiquitin at 1.70-1.98 Å resolution". This sounds very confusing and they should clearly state that they are talking here about different crystal forms and two complexes with di and one with tetraubiquitin.

In the Introduction the authors say that the mechanism by which DUBs downregulate NF- κ B activation remains elusive - this seems a bit disingenuous as one would assume them to downregulate by virtue of their DUB activity - and is the mechanism of action of CYLD. Please rephrase.

This may seem a bit picky but the authors state concerning Figure 1A that CYLD "strongly" suppresses NF- κ B activation, which is only about 2-fold over Cezanne which has no effect and then say that the CYLD C601 mutants "slightly" enhances activity - which is also roughly 2-fold. Please be consistent with these types of statements.

How have the authors controlled for the level of transfection and made sure that any differences seen with different constructs are not due to different levels of transfection and/or protein

expression? It would be very useful if in all the Figures that showed NF- κ B activation assays the authors also showed total protein content of the protein under investigation, ideally with the same tag.

When referring to Figure S1 please specify which panel (A, B, C).

The authors should check if the heat of dilution has been subtracted from the fit in Figure 2C - it doesn't look that way. The authors should also use this titration to explicitly say what the stoichiometry of the interaction is.

I don't think the statement that linear diUb binds more strongly to ZF7x3 is correct - it just binds 3 times as many molecules as there are 3 binding sites but I don't think there are any avidity effects.

I'm not convinced that showing helices as cylinders in Figure S9 is very helpful - they look more like light sausages, sometimes with strange cuts (see NEMO in S9B).

1st Revision - authors' response

22 July 2012

First of all, we would like to thank you for generously allowing us to revise our manuscript. We also appreciate the suggestions and concerns raised by the referees, which have improved the manuscript. In light of their comments and suggestions, we have revised the manuscript as follows. We believe that our manuscript has been considerably improved by the incorporation of the new experiments and is now acceptable for publication in *EMBO J*.

According to your suggestion, we reduced the number of Supplementary Figures by incorporating important data, including a structural comparison of ubiquitin-binding domains, into the main manuscript, and appropriately adjusted the contrast of gels and blots. In addition, we included PDB accession codes in the revised manuscript (3VUW, 3VUX and 3VUY), and added relevant citations that were missing in the previous version of our manuscript.

Referee #1

1) It has been controversial whether A20 requires its DUB activity to inhibit NF- κ B. Overexpression of A20 mutants in 293-T cells may yield misleading results since these cells have high basal levels of A20 which may interact with the overexpressed forms of A20 and influence NF- κ B activation. Therefore, some of the key results in Fig. 1 need to be repeated in an A20-null (either knockout or siRNA knockdown) background. The authors also need to show expression of transfected proteins (i.e. Figs. 1B-F, 3B).

As pointed out by the reviewer, it is crucial to confirm the effects of various A20 mutants on LUBAC-mediated NF- κ B activation in A20-null cells. As shown in the new Fig. 1G, we performed luciferase assays in A20^{-/-}-MEFs. Due to the difficulty in transfecting MEFs, LUBAC expression induced only about a 2-fold increase in NF- κ B activity. As observed in HEK293T cells (Fig. 1B-E), A20 WT and ZF7-containing mutants, such as C103A, ZF1-7, ZF4-7, and ZF4CA, but not ZF7-deleted and dysfunctional mutants, suppressed LUBAC-induced NF- κ B activation. These results confirmed the functional significance of A20 ZF7 in the regulation of LUBAC-mediated NF- κ B activation in A20-null cells. We also indicated the expression levels of the transfected proteins in the new Figs. 1 and 3.

3) *Overexpression of LUBAC (Fig. 1B, 4A) and TRAF6 (Fig. 1E) is highly artificial. The authors should also repeat these experiments with stimulation (i.e. TNF or IL-1).*

We agree that it is important to investigate whether A20 ZF7 plays a role during proinflammatory cytokine stimulation, such as by TNF- α . We examined the effects of A20 WT and mutants on TNF- α -induced NF- κ B activation in HEK293T cells (Fig. 1F), and confirmed that A20 ZF7 is indispensable for the suppression of TNF- α -induced NF- κ B activation. Moreover, we examined the effect of A20 ZF7 on TNF- α -induced NF- κ B activation in *A20^{-/-}*-MEFs (Fig. 1H), *SHARPIN*-ablated *cpdm*-MEFs (Fig. 1I), and *HOIL-1L^{-/-}*-MEFs (Fig. 1J). These results further supported the physiological significance of A20 ZF7 in the regulation of TNF- α -induced NF- κ B activation. Importantly, the inhibitory effects of A20 WT and ZF1-7 were attenuated in LUBAC-ablated *cpdm*- and *HOIL-1L^{-/-}*-MEFs, indicating that A20 ZF7 plays an inhibitory role downstream of LUBAC in the TNF- α -mediated NF- κ B pathway.

4) *Fig. 4A lacks the wild-type A20 control. Supp. Fig. 10 lacks controls with the A20 mutants alone. Supp. Fig. 10 should also have positive controls for ABIN-1 and TAX1BP1 (i.e. inhibition of IL-1-induced NF- κ B).*

Since we have shown the inhibitory effect of A20 WT in Fig. 1B (previous Fig. 1A), we did not show the control in the previous Fig. 4A. As requested by the reviewer, we indicated the effect of A20 WT in the new Fig. 5A. As positive controls for the effects of ABIN-1 and TAX1BP1, we performed luciferase reporter assays targeting TRAF6-induced NF- κ B activation. As shown in the new Fig. 5B, as compared with LUBAC-induced NF- κ B activation, ABIN-1 and TAX1BP1 showed higher inhibitory effects on TRAF6-induced NF- κ B activation, although the expression of ABIN-1 and TAX1BP1 by themselves showed weak suppressive effects in both cases.

5) *Generally, more experiments are needed with endogenous proteins in the manuscript to confirm overexpression results (i.e. co-IPs for endogenous A20, HOIP, HOIL-1L, Sharpin and NEMO binding). Stimulation of cells with TNF for various time-points will reveal if these interactions are stimulus-dependent.*

We agree with these comments. At first, we investigated the formation of the TNFR signalling complex by FLAG-TNF- α pulldown experiments in *A20^{+/+}*- and *A20^{-/-}*-MEFs (Fig. 6A). We confirmed that A20, as well as LUBAC (HOIP) and IKK (NEMO), are recruited to the TNFR signalling complex upon TNF- α stimulation in *A20^{+/+}*-MEFs. The association of LUBAC and NEMO (IKK) with TNFR was enhanced by the ablation of A20. Moreover, in addition to the overexpression experiments, we examined the endogenous association of A20 with LUBAC (HOIP) in *A20^{+/+}*- and *A20^{-/-}*-MEFs stimulated with TNF- α , and confirmed the endogenous association of A20 with LUBAC upon TNF- α stimulation (Fig. 6E). These results clearly indicated that A20 physiologically associates with LUBAC and NEMO in the TNFR signalling complex upon TNF- α stimulation.

6) *In the absence of A20 (either knockdown or genetic deficiency), is linear ubiquitination of NEMO enhanced and more persistent after TNF stimulation? The authors should examine endogenous linear ubiquitination of NEMO using the linkage-specific linear Ub antibody previously used by the authors (Tokunaga et al 2009 Nat. Cell Biol. 11: 123-32).*

As shown in the new Fig. 6C, we analyzed the total ubiquitination of NEMO in *A20^{+/+}*- and *A20^{-/-}*-MEFs stimulated with TNF- α , since it is important to first investigate the entire ubiquitination status of NEMO upon TNF- α stimulation. We found that the total polyubiquitination of NEMO was not drastically affected by the ablation of A20, consistent with our data showing that A20 neither cleaves linear polyubiquitin (Supplementary Fig. S1) nor inhibits the LUBAC E3 activity

(Supplementary Fig. S3). As shown in Fig. 6A, the polyubiquitination of TNFR-associated RIP1 was enhanced in TNF- α -treated *A20*^{-/-}-MEFs, although the linkage between the ubiquitins is unknown. It will be crucial to investigate the precise ubiquitination status of NEMO upon TNF- α stimulation in future studies.

7) As mentioned above, a weakness of the paper is that the mechanism of A20 inhibition of LUBAC-induced NF- κ B activation remains unknown. Although ZF7 binds to linear Ub chains and recruits A20 to LUBAC-IKK, how does A20 actually inhibit NF- κ B? The authors should at least discuss potential models.

In the revised manuscript, we performed additional experiments to elucidate the molecular mechanism of A20 inhibition of LUBAC-induced NF- κ B activation. We found that the linear ubiquitin-binding activity of A20 ZF7 contributes to the recruitment of A20 to TNFR upon TNF- α stimulation (Fig. 6A, B), and is important for the complex formation with LUBAC and NEMO (Fig. 6D). We also found that the A20 Δ ZF7 and A20 N772K/E781D mutants, which lack linear ubiquitin binding activity, showed impaired recruitment to TNFR (Fig. 6B) and hyper-activation of NF- κ B (Fig. 4F). Based on these results, we proposed a model for the suppression of LUBAC-mediated NF- κ B activation, in which TNF- α stimulation causes A20 to be recruited to the TNFR signalling complex via the interaction between A20 ZF7 and linear polyubiquitin on NEMO and RIP1, facilitating the dissociation of LUBAC and NEMO from TNFR.

Referee #2

Major points:

1. In general: from the data provided in the manuscript, it is clear that A20 inhibits LUBAC-mediated NF- κ B activation by binding to linear ubiquitin chains in the system they used. The authors examined the effects of different A20 mutants on LUBAC-induced NF- κ B activation by using NF- κ B reporter assay in HEK293T cells overexpressing A20 wt or mutants (Fig 1 A-E, Fig 3D, Fig 4A, Supp Fig 4A, Supp Fig 10 A and B). The major concern is that whether the negative effects observed in the manuscript is just dependent on the overexpression of linear-ubiquitin binding proteins especially by looking at the results of Supp Figure 4A in which authors used tandem repeats of ZF7X2 and ZF7X3. In this figure, single ZF7 alone has almost no effects on LUBAC-induced NF- κ B activation but the effects become drastic when ZF7X2 or ZF7X3 was used in the assay. Even though they showed that ABIN-1 overexpression has little effects on LUBAC-induced NF- κ B activation (Supp Figure 10A), the authors need to show the specific role of A20-ZF7 by comparing with other linear-ubiquitin binding domains, such as NEMO-UBAN X1, X2 or X3 or ABIN-UBANx1, X2 or X3 using the same technique as Supp Fig 4A.

We thank the reviewer for this crucially important comment. According to the reviewer's suggestion, we constructed human NEMO-UBANX1, X2 and X3, and human ABIN-1-UBANx1, and then examined their inhibitory effects on LUBAC-induced NF- κ B activation (Fig. 5F). We found that overexpression of NEMO-UBANx1, X2 and X3 as well as ABIN-1-UBANx1 also showed inhibitory effects on LUBAC-induced NF- κ B activation (Fig. 5F). However, unlike full-length A20, the expression of full-length ABIN-1 showed modest inhibitory effects on LUBAC-induced NF- κ B activation (Fig. 5A). The present crystal structure indicated that full-length A20 can bind linear polyubiquitin in a manner similar to that observed in A20 ZF7 alone (Supplementary Fig. S4A). We concluded that, although the binding of UBDs to linear ubiquitin chains is sufficient for the suppression of LUBAC-mediated NF- κ B activation, full-length ABIN-1 has weaker inhibitory effects on LUBAC-mediated NF- κ B activation than ABIN-1 UBAN alone, partly because full-length ABIN-1 binds linear ubiquitin chains less effectively than ABIN-1 UBAN alone, due to steric hindrances.

2. *In general: the effects of full-length A20 mutants, which do not interact with linear-ubiquitin chains were exclusively tested in an overexpression system. The authors also need to show the role of A20-ZF7 by generating the A20-ZF7 mutants (N772 mutant and E781 mutant) expressing cells in the background of A20-knock out or A20-knock down. These cells then need to be stimulated by TNF- α (and/or other cytokines) and NF- κ B activation needs to be examined.*

According to the reviewer's suggestion, we constructed A20^{-/-}-MEFs expressing A20 WT, Δ ZF7 and N772K/E781D mutants at similar levels to A20 in A20^{+/+}-MEFs (Fig. 4E), and examined I κ B α degradation and re-accumulation upon TNF- α stimulation (Fig. 4F). We found that the expression of A20 WT, but not those of the Δ ZF7 and N772K/E781D mutants, restored the regulated NF- κ B activation (Fig. 4F), supporting the physiological significance of A20 ZF7 for the suppression of TNF- α -mediated NF- κ B activation.

3. *In general, following up the question above (#2): one of the major points unclear in the manuscript is the actual role of A20-ZF7 binding to linear ubiquitin chain. If A20 does not catalyze linear ubiquitin chains, either free chains or the ones on NEMO, what does A20 do by binding to linear ubiquitin chain? The authors showed that A20 gets recruited into the LUBAC-IKK complex however it is still not yet clear how it controls the NF- κ B activation. Does A20 disturb the complex formation of positive regulator of NF- κ B downstream or inhibit IKK activity or any downstream molecules? This point is not clear and needs to be addressed by using the A20 mutant cells (as suggested in #2), analyzing the TNFR complex formation or TAK1-mediated IKK activation as the authors discussed in the text in page 18.*

As suggested by the reviewer, we investigated the TNFR complex formation by co-immunoprecipitation. We first confirmed that, in A20^{+/+}-MEFs, A20 is recruited to TNFR, together with LUBAC and NEMO, upon TNF- α stimulation (Fig. 6A). The recruitment of LUBAC and NEMO to TNFR and the polyubiquitination of RIP1 were enhanced and sustained in A20^{-/-}-MEFs (Fig. 6A), indicating that A20 facilitates the dissociation of LUBAC and NEMO from TNFR under physiological conditions. Furthermore, we found that the A20 Δ ZF7 and A20 N722K/E781D mutants exhibit defects in the regulation of TNF- α -induced NF- κ B activation (Fig. 4F) and in the recruitment to TNFR after TNF- α stimulation (Fig. 6B). Thus, we proposed a model, in which A20 is recruited to the TNFR signalling complex via the interactions between A20 ZF7 and linear polyubiquitin on NEMO and RIP1, facilitating the dissociation of LUBAC and NEMO from TNFR under physiological conditions.

4. *Figure 1F: The authors tried to show in this figure that the effect of A20-ZF7 is dependent on LUBAC by using wt and HOIL-1L deficient MEFs. It is clear that ZF1-7 is required for the inhibitory effects on NF- κ B in wt MEFs but not in HOIL-1 KO MEFs. On the other hand, these data lead to a question if HOIL-1L deficiency in MEFs lead complete loss of linear ubiquitination upon TNF- α stimulation since Sharpin should still plays a role in this condition. If Sharpin also plays a role in linear ubiquitination as the authors previously published (Tokunaga et al, 2011), ZF1-7 in HOIL-1L deficient cells should still inhibit certain extent of NF- κ B activation. The authors need to clarify this point by comparing with Sharpin deficient cells and/or Sharpin/HOIL-1L double deficient cells.*

As described previously (Tokunaga et al. Nat Cell Biol 2009; Tokunaga et al. Nature 2011), two components of LUBAC, HOIP-HOIL-1L and HOIP-SHARPIN, form trace amounts of a high molecular weight complex in *cpdm*- and *HOIL-1L*^{-/-}-MEFs, respectively, and thus residual TNF- α -induced NF- κ B activation was observed in both MEFs. We examined the inhibitory effects of A20 WT and mutants in *SHARPIN*-ablated *cpdm* MEFs (Fig. 1I). As observed in HEK293T cells (Fig. 1F), A20^{-/-}-MEFs (Fig. 1H) and *HOIL-1L*^{+/+}-MEFs (Fig. 1J), the expression of A20 WT and ZF1-7 suppressed TNF- α -induced NF- κ B activation in *SHARPIN*^{+/+}-MEFs, whereas their inhibitory

effects were attenuated in *cpdm*-MEFs. In *cpdm*-MEFs, the A20 mutant lacking ZF7 (Δ ZF7 and ZF1-6) showed no inhibitory effect (Fig. 1I). Thus, the inhibitory spectrum in *cpdm*-MEFs was similar to that in *HOIL-1L*^{-/-}-MEFs. We further tried luciferase reporter assays in *cpdm*-MEFs with an siRNA knockdown of *HOIL-1L*. *SHARPIN/HOIL-1L* double-deficient cells showed potent inhibition of TNF- α -induced NF- κ B activation (Tokunaga et al. Nature 2011). Since most of these cells died within 6 h after TNF- α -treatment, we were unable to perform luciferase reporter assays in the *SHARPIN/HOIL-1L* double-deficient cells. Collectively, these results suggested that A20 plays an inhibitory role downstream of LUBAC in the TNF- α -induced NF- κ B activation pathway.

Minor point:

1. In general: Authors need to distinguish clearly in the text if the effects of A20 on the NF- κ B activation is LUBAC mediated or not. In some sentences, it is only described as 'A20 suppressed NF- κ B activation independently to the DUB catalytic activity' but it should be written as 'A20 suppressed LUBAC-induced NF- κ B activation independently to the DUB catalytic activity'. This is critical since A20 was shown to have negative effects on the NF- κ B signalling through RIP1 in a distinct mechanism.

We apologize for the insufficient specification of the NF- κ B activation pathway. In this revised manuscript, we carefully described the NF- κ B activation pathway.

Referee #3

Major points

In Figure S4A the authors show that the isolated ZF7 has hardly any effect on NF- κ B activation, which the authors explain by its low expression. However, in Figure 1C the authors see a relatively strong effect upon expression of ZF6-7. Is this really purely due to levels of protein expression? If yes, the authors need to show protein expression levels in all activation assays to support this statement.

As shown in the new Figs. 1D and 5C, the expression of ZF6-7, but not the single ZF7, was detectable in HEK293T cell lysates. Moreover, in this revised manuscript, we showed the expression levels of the A20 mutants as clearly possible.

What point are the authors trying to make with the paragraph "TRAF6 is an E3 ligase..." What is the difference between TRAF6 and LUBAC mediated NF- κ B activation? Please give the reader a bit more information, are the authors trying to imply that this might be due to different ubiquitin chains being involved? If yes, have the paragraph later and say so explicitly. If the authors think there are other reasons they should say so as well.

We apologize for the ambiguous description. We have shown that LUBAC extensively generates M1-linked linear polyubiquitin chains, but not other Lys-linked chains (Kirisako, T. et al., EMBO J. (2006)), and induces the canonical NF- κ B pathway (Tokunaga, F. et al., Nat. Cell Biol. (2009); Tokunaga, F. et al., Nature (2011)). In contrast, TRAF6 is an E3 ligase that predominately produces K63-linked polyubiquitin chains, but not linear chains, and also activates the canonical NF- κ B pathway. We compared the significance of A20 ZF7 in the LUBAC- and TRAF6-induced NF- κ B activation pathways, to represent these ubiquitin chain-types. In this revised manuscript, we tried to clarify the differences in the ubiquitin linkages involved in the LUBAC- and TRAF6-mediated pathways, as described on page 8.

Given the low level of input of K48 chains versus K63 chains in Figure 5C I'm not convinced that the residual binding to K48 chains shown is not almost as strong as to K63 chains in comparison. The point I'm trying to make is that A20 ZF7 binding to linear chains is convincing and

specific whereas the binding to K63 chains is in my opinion just an unspecific artifact, probably exaggerated by doing pulldown assays and hence doesn't require the amount of discussion it gets on page 17.

We agree with the reviewer's comment that we should focus on the specific binding of A20 ZF7 to the linear chains. In this revised manuscript, we deleted the confusing, previous Supplementary Fig. S5, and shortened the discussion about the binding of A20 ZF7 to the "long" K63-linked polyubiquitin.

The description of the different crystal forms and their packing is rather confusing and I seriously doubt that a non-structural biologist will understand the description of the filaments. The authors need to explain better how the ZF is capable of stitching the diubiquitin together to form these filaments. What type of polyubiquitin chains have been used for the EM pictures - long chains? The authors indicate that it is unlikely that this filament formation plays a role in vivo - if this is the case I think it might be better to remove the EM pictures as this just confuses the story. On the other hand, if they think there might be something to it they have to investigate it in more detail - for example make mutants that should disrupt filament formation and then test them in NF- κ B assays and test other domains specific for linear chains such as the UBAN of NEMO for their ability to induce filament formation.

In the revised manuscript, we added a description about the different crystal forms in the main text, and added the new Supplementary Fig. S4A (schematic drawings of the crystal packing in the diubiquitin complex and the tetraubiquitin complex). As pointed out by the reviewer, filament formation is confusing and its physiological significance remains unclear. We removed the description about the filament formation, and the previous Supplementary Fig. S8.

Why don't the authors show the structure of the ZF bound to tetraubiquitin? Do 2 ZF bind to tetraubiquitin? Please add this as a supplemental Figure.

Both of the crystals obtained in the presence of diubiquitin or tetraubiquitin comprise an A20 ZF7-diubiquitin unit with a virtually identical conformation, in which one A20 ZF7 molecule binds between two M1-linked ubiquitin molecules. Therefore, as a representative, we showed the one unit of A20 ZF7 complexed with diubiquitin in the tetraubiquitin complex. We added a description about crystal packing in the main text, and added the new Supplementary Fig. S4A (schematic drawings of the crystal packing in the diubiquitin complex and the tetraubiquitin complex).

How do the authors know that they have potassium bound and what is the role of this ion in this structure? Isn't that highly unusual? This needs to be further explained.

The potassium ion is probably derived from the crystallization buffer, and is coordinated by the main-chain carbonyl groups of Cys762, Ala764 and Cys767 in a loop region, suggesting that it helps maintain the loop conformation (new Fig. 3B). Potassium binding to A20 ZFs is unusual, since A20 ZF4 (PDB: 3OJ3) has a similar loop, but does not bind a potassium ion. However, the physiological relevance of the bound potassium ion is currently unknown.

How can Cys767 which coordinates the Zn²⁺ ion form a salt bridge? I think what the authors are referring to is at most a hydrogen bond.

It seems likely that Cys767 exists as a negatively charged thiolate ion by the coordination of its sulfur atom to the Zn²⁺ ion, and thus Cys767 interacts with the positively charged side chain of Arg72 of the distal ubiquitin.

The inset showing the Zn²⁺ and K⁺ coordination is not very clear. This Figure should be redrawn showing much more clearly which residues coordinate what. A schematic might be more useful here than the actual structure.

Similarly, I don't think Figure 3B shows very well the "the recognition of a hydrophobic patch by shape complementarity..."

We made the new Fig. 3B, showing the overall structure of A20 ZF7, viewed from a different angle (in stereo), to clearly depict the coordination of the Zn²⁺ and K⁺ ions. In addition, we revised the previous Fig. 3B (new Fig. 3D), to clearly show the shape complementarity between A20 ZF7 and diubiquitin.

How have the authors ensured that the mutants that lost their ability to bind ubiquitin are not unfolded? This should be tested by CD spectroscopy or similar.

We analyzed the purified A20 ZF7 mutants by size-exclusion chromatography. Like WT A20, all of the mutants eluted as a single peak from the size-exclusion column (new Supplementary Fig. S5), suggesting that the mutants are correctly folded.

Why does the N772A mutant which completely lost the ability to bind linear di-Ub still suppresses NF-κB activation nearly as well as wt? This absolutely doesn't make sense and needs to be explained.

The GST-tagged A20 N772A mutant apparently lacked the ability to bind linear diubiquitin in GST pulldown assays (Fig. 3E), while luciferase reporter assays showed that the full-length A20 N772A mutant exhibits modest inhibitory effects on LUBAC-induced NF-κB activation (Fig. 3F). Similarly, the N772K and E781D mutants apparently lacked the ability to bind linear diubiquitin in GST pulldown assays (Fig. 4C), while these mutants caused partial suppression of LUBAC-induced NF-κB activation (Fig. 4A). We assume that these discrepancies are partly derived from the differences in their affinities for linear ubiquitin chains, which were undetectable in GST pulldown assays. To test this hypothesis, we examined the binding of the N772K and E781D mutants to linear tetraubiquitin and K63-linked tetraubiquitin, using GST pulldown assays. As shown in the new Fig. 4D, the N772K and E781D mutants both apparently lacked linear ubiquitin binding ability, while a longer exposure revealed the faint binding of the E781D mutant to linear tetraubiquitin, indicating the difference in their affinities for the linear chains, which is correlated with their abilities to suppress LUBAC-induced NF-κB activation in luciferase reporter assays. We concluded that the differences in the ubiquitin-binding abilities of the A20 mutants, which are undetectable in GST pulldown assays, at least partly lead to the apparent discrepancies between the GST pulldown and luciferase reporter assay results.

I'm very confused about the last paragraph of Results which shows an interaction between the HOIP subunit of LUBAC and ZF7. How does this fit with the ubiquitin binding property of this ZF? Are the authors suggesting that it can bind both, HOIP and linear ubiquitin chains? I think this raises a fundamental question about the model suggested in this manuscript: do the authors suggest that the linear ubiquitin-binding properties of A20 ZF7 will sequester linear chains away from the UBAN of NEMO or alternatively "block" linear chains attached to NEMO and thereby inhibit activation of NF-κB or do the authors think that the ZF binds HOIP and thereby somehow interferes with NF-κB activation. It appears that in the discussion on page 18 the authors try to keep everybody happy by saying that this ZF can do whatever required - I find this rather frustrating and would like the authors to present a more explicit model that is supported by their data.

Our immunoprecipitation data indicated that A20 associates with HOIP either directly or indirectly, when they are overexpressed in HEK293T cells. However, as pointed out by the reviewer, this is confusing and its physiological significance remains unclear. In this revised manuscript, we deleted the overexpression data in HEK293T cells. Instead, we added new data showing the TNFR signalling complex formation in $A20^{+/+}$ - and $A20^{-/-}$ -MEFs (Fig. 6A, B) and the endogenous A20-LUBAC interaction upon TNF- α stimulation (Fig. 6E). We found that LUBAC, IKK (NEMO) and A20 are incorporated into the TNFR signalling complex after TNF- α stimulation (Fig. 6A) and the binding of A20 ZF7 to linear ubiquitin chains is important for the recruitment of A20 to TNFR (Fig. 6B). We also found that the endogenous A20-LUBAC interaction was increased upon TNF- α stimulation (Fig. 6E). The recruitment of LUBAC and NEMO to TNFR was enhanced in the absence of A20 (Fig. 6A), suggesting that the recruitment of A20 to TNFR is important for the downregulation of NF- κ B activation. Moreover, we found that the overexpression of tandem conjugates of A20 ZF7, NEMO UBAN and ABIN-1 UBAN suppressed LUBAC-mediated NF- κ B activation (Fig 5C, F), indicating that the binding of UBDs to linear ubiquitin chains is sufficient for the suppression of LUBAC-mediated NF- κ B activation. Taken together, these endogenous and overexpression studies indicated that the recruitment of A20 to TNFR via the interaction between A20 ZF7 and linear polyubiquitin is important for the downregulation of LUBAC-mediated NF- κ B activation.

Have the authors looked at binding of ZF7 to monoUb? Given that it is not the Ub-Ub linkage that is recognized by the ZF, monoUb should bind just with lower affinity. This experiment should be included, ideally by ITC as this would also give the stoichiometry of the interaction

According to the reviewer's suggestion, we examined the binding of A20 ZF7 to monoubiquitin by ITC. As with the K48-linked and K63-linked diubiquitins, the heat changes were too small to determine a K_d value for monoubiquitin (new Fig. 2C). These results further support the notion that A20 ZF7 selectively recognizes linear diubiquitin through simultaneous interactions with the proximal and distal ubiquitin moieties in the linear chains.

Minor points

The authors say in the abstract and introduction that the "determined the structures of A20-ZF7 in complex with linear diubiquitin at 1.70-1.98 Å resolution". This sounds very confusing and they should clearly state that they are talking here about different crystal forms and two complexes with di and one with tetraubiquitin.

We crystallized A20 ZF7 in the presence of either diubiquitin or tetraubiquitin under similar conditions, and determined the crystal structures. The structures revealed two different crystal forms, Form I (in the presence of diubiquitin) and Form II (in the presence of diubiquitin or tetraubiquitin), and an A20 ZF7-diubiquitin complex with an essentially identical conformation was commonly observed in both Forms I and II. Although these A20 ZF7-diubiquitin complexes are crystallographically identical, the electron density for the linkage is most clearly observed in the tetraubiquitin complex, due to the higher occupancy in the linkage region. In the revised manuscript, we included a description about these features, and added the new Supplementary Fig. S4A (schematic drawings of the crystal packing in the diubiquitin complex and the tetraubiquitin complex).

In the Introduction the authors say that the mechanism by which DUBs downregulate NF- κ B activation remains elusive - this seems a bit disingenuous as one would assume them to downregulate by virtue of their DUB activity - and is the mechanism of action of CYLD. Please rephrase.

We thank you for this important comment. On page 5 in the Introduction, we rephrased the sentence as “However, it remains elusive how DUBs physiologically downregulate LUBAC-mediated NF- κ B activation”.

This may seem a bit picky but the authors state concerning Figure 1A that CYLD "strongly" suppresses NF- κ B activation, which is only about 2-fold over Cezanne which has no effect and then say that the CYLD C601 mutants "slightly" enhances activity - which is also roughly 2-fold. Please be consistent with these types of statements.

As described in page 6, we deleted such non-quantitative statements.

How have the authors controlled for the level of transfection and made sure that any differences seen with different constructs are not due to different levels of transfection and/or protein expression? It would be very useful if in all the Figures that showed NF- κ B activation assays the authors also showed total protein content of the protein under investigation, ideally with the same tag.

As described in the first part of the Major Points, in this revised manuscript, we showed the expression levels of A20 WT and mutants, together with a loading control of tubulin, as much as possible. Since A20 WT and mutants were tagged with FLAG, their expression levels are comparable within the Figures.

When referring to Figure S1 please specify which panel (A, B, C).

Thank you for the comment. We specified the panels in the revised manuscript.

The authors should check if the heat of dilution has been subtracted from the fit in Figure 2C - it doesn't look that way. The authors should also use this titration to explicitly say what the stoichiometry of the interaction is.

We subtracted the heat of a dilution control (A20 ZF7 into the buffer) from the sample titration data, and calculated a K_d value and the stoichiometry (new Fig. 2C).

I don't think the statement that linear diUb binds more strongly to ZF7x3 is correct - it just binds 3 times as many molecules as there are 3 binding sites but I don't think there are any avidity effects.

A previous study showed that a tandem conjugate of ubiquitin-binding domains has increased affinity for ubiquitin (EMBO Rep. 10, 1250-1258, (2009)). However, as pointed out by the reviewer, we did not investigate the dissociation constants of A20 ZF7x3 to linear diubiquitin. In this revised manuscript, we changed the description to “A20 ZF7x3 binds larger amounts of linear diubiquitin, as compared with A20 ZF7 alone” on pages 16-17, as an appropriate interpretation for the new Fig. 5D.

I'm not convinced that showing helices as cylinders in Figure S9 is very helpful - they look more like light sausages, sometimes with strange cuts (see NEMO in S9B).

We made the new Fig. 7 (previous Supplementary Fig. S9), in which the helices are depicted by a ribbon representation.

We greatly appreciate your help and the comments by the referees concerning the improvement of this paper. We believe that the revised manuscript is now suitable for publication in *EMBO Journal*.

Acceptance letter

03 August 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections from their side. We are therefore ready to accept your article for publication in The EMBO Journal at this point.

You shall shortly receive a formal letter of acceptance, describing details on the further proceedings. Importantly, in case you have not already sent the relevant copyright and page charge authorization forms, please do so as early as possible in order to avoid delays in the production process.

Thank you once again for this contribution to The EMBO Journal and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
The EMBO Journal

Referee #1

In my opinion, the revised manuscript is significantly improved and suitable for publication in EMBO J.

Referee #3

The authors have carried out many additional experiments to respond to the points raised by the reviewers. I'm satisfied that all of my concerns have been adequately addressed.