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NEMO recognizes K63-linked polyubiquitin chains through a new bipartite ubiquitin binding domain

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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 11 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as interesting and (potentially) important in principle and referees 2 and 3 offer strong support for publication of the study here. Referee 1 is more critical and feels that the evidence presented is not strong enough to fully justify the conclusions drawn - at least at this stage of analysis. Given the interest expressed by all three referees and given the strong positive view of referees 2 and 3 we should be happy to consider a revised manuscript in which you need to address (or respond to) the referees' criticisms in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript authors aim to show that the ubiquitin binding C-terminal zinc finger (ZF) and ubiquitin binding NOA/CoZi/UBAN/NUB domain of NEMO cooperate to enhance the affinity and specificity of NEMO to bind K63 linked polyubiquitin chains. To this end using fluorescence spectroscopy, pull-down assays and functional luciferase assays using several ZF chimera proteins (with ubiquitin binding ZF domains derived from Optineurin, ABIN2,WRNIP1 and Rabex5) they conclude that the C-terminal ZF of NEMO (or any of the above mentioned proteins) is sufficient to bind polyubiquitin chains and restore Nf-kB signaling in NEMO deficient cell lines.

Several groups showed that the NEMO NOA/CoZi/NUB/UBAN domain has a preference to K63-linked polyubiquitin chains in vivo. Very recently, Lo et al., 2009, Rahihi et al., 2009 and Komander et al., 2009 provided structural and functional evidence that UBAN binds to linear ubiquitin chain with two monomers with at least 100X stronger affinity than to the K63-linked diubiquitin. Here the authors provide interesting data suggesting a bipartite cooperation of two ubiquitin binding domains of NEMO which enhance K63-linked polyubiquitin binding, but do not affect the binding to the linear ubiquitin chains.

The roles of C-terminal ZF of NEMO is interesting because the mutations in this region also reported in relatively severe EDA-IDs. Possibly the ubiquitin binding activity of ZF might be critical. However, the reviewer cannot agree with the authors' opinion that ZF is co-ordinatively involved in K63 chain, but not to K48 and linear, recognition. It is unclear why there would be a specificity for K63 polyubiquitin chains. Moreover, there are several technical issues in the manuscript which undermines the importance and novelty of the conclusions made.

General Comments:

1) By fluoresence spectroscopy the authors showed that the entire C-terminal of NEMO (NOAZ, including the NOA and C-terminal ZF) increased the affinity of NEMO to both K63-linked and K48-linked polyubiquitin chains, as compared with the NOA domain alone, whereas the affinity for the linear chains did not change. The authors used only the tetramer in the case of linear polyubiquitin chains, but they used 3-7 ubiquitin monomers in K63 and K48 chains. This is a problem because the length of the ubiquitin chains could be very important for the binding with both NOA and ZF. It is likely that the linear tetraubiquitin is too short, while longer K63-linked chains, for example Ub6 and Ub7 of the Ub3-7 mixture, are long enough to reach both ubiquitin binding sites. Therefore the authors must compare linear, K63-, and K48 linked ubiquitin chains of the same length rather than the mixture of certain chain lengths (3 to 7 in their case) to have a fair comparision between different linkages. Such experiments should be performed with a range of ubiquitin length from Ub3 to Ub7 (individually).

2) The authors should examined affinity of linear polyubiquitin binding to ZF (not NOAZ).

3) The authors should also explain binding of K48 polyubiquitin chains to NOA and NOAZ in the light of their potential function in NEMO.

4) The authors claim that the linker (which varies among NEMO, Optinuerin and ABIN2) is dispensable. More experiments need to be done to support this statement.

5) Functional studies presented in this manuscript are very limited and poorly performed. For example, in experiments using NEMO efficient cells, the expression level of NEMO mutants is very important. The expression level of NEMO and its mutants must be at the comparable level of endogenous level (namely the expression level of parent cells or other MEFs). When overexpressed we cannot see effects of subtle changes provoked by mutations of NEMO.

6) Tables 1 and 2: Authors should use the same constructs in all the experiments in the manuscript: isolated ZFs, CC2-LZ, NOAZ various chimeras etc. for the sake of intrinsic consistency. It would also be beneficial to their story if they performed the fluorescence spectroscopic analyses with K63,

K48 and linear- diUb chains in order to confirm or challange the recently published studies (Lo et al., 2009, Komander et al., 2009 and Rahighi et al., 2009).

7) Authors should use WT forms of proteins to create chimeras in their luciferase assays as controls, perform appropriate quantification methods and statistical analyses when making conclusions and provide transfection controls in the same cell types used for the study.

8) Data should be explicitly shown for the complementation experiments of F312W mutation (mentioned in page 8) since the F312A mutation has been shown to affect ubiqitin biding by several groups.

Referee #2 (Remarks to the Author):

Ubiquitin, a 76 amino acid globular polypeptide targets numerous proteins for degradation, translocation or activation. Ubiquitination plays an essential signaling role in innate immunity, acquired immunity, and tolerance. NEMO (IKK) is a ubiquitin binding protein, that transduces multiple immune responses to activation of NF-kB. In this manuscript, Fontan et al, found that a bipartite ubiquitin domain in NEMO, NOA-ZF, binds to K63-linked ubiquitin chain: an event, required for downstream NF-kB activation. Their findings are important, novel and exciting.

Minor point:

Though the authors perform reconstitution study with NEMO mutants in JM4.5.2 and MEF cells, recontituted NEMO expression data is missing (Fig. 2 and 4). This needs to be provided.

Referee #3 (Remarks to the Author):

It is now firmly established that NEMO is critically important for NF-kB activation, but the mechanism by which NEMO regulates IKK is still not very well understood. Accumulating evidence has suggested that the binding of NEMO to K63 polyubiquitin chains and/or ubiquitination of NEMO are important for its function. However, some recent studies have also argued that conjugation of NEMO by linear polyubiquitin chains or the binding of NEMO to linear chains is the physiological mechanism that regulates IKK activation. The argument for the role of linear polyubiquitin chains in NEMO signaling is largely based on structural studies of an isolated ubiquitin-binding domain of NEMO, termed UBAN, NUB, CoZi or NOA. A second ubiquitin-binding domain of NEMO has recently been shown to reside in the C-terminal zinc-finger (ZF) domain of NEMO. Laplantine et al now present convincing evidence that both NOA and ZF domains are important for the full activity of NEMO. Strikingly, their quantitative measurements of ubiquitin binding by NOA and NOA+ZF show that the ZF domain increases the affinity of binding to K63 polyubiquitin chains by ~100 fold, and the specificity of K63 vs K48 ubiquitin chains by 30 fold. In contrast, the ZF domain does not contribute to binding of NEMO to linear polyubiquitin chains. Functional studies show that the ubiquitin-binding function of the ZF domain is important for NF-kB activation in complementation experiments. The authors also carried out domain swapping experiments to demonstrate that the major function of the NOA and ZF domains is to bind K63 polyubiquitin chains.

This is an excellent study that combines quantitative Ub-binding affinity measurements with functional NF-kB assays and the results provide very strong evidence that the bipartite ubiquitin-binding domains of NEMO and its binding to K63 polyubiquitin chains are important for IKK activation. These results should clarify the confusions generated by studies using an isolated ubiquitin-binding domain from NEMO. This work would be of interest to a large community of scientists interested in NF-kB, signaling and ubiquitin. I highly recommend publication of this paper in EMBO jounal.

Minor points:

1) Although the authors' data that deletion or mutation of the C-terminal ZF impairs NF-kB activation support an important role of K63 chains rather than linear chains, Rahighi et al (2009)

showed that mutations within the NOA domain (R316/R319/E320) that disrupt binding to linear but not K63 Ub chains also impair NF-kB activation, arguing for an important role of linear Ub chain binding. To clarify this issue, the authors should measure the affinity of NOA and NOAZ carrying the same mutations for K63 vs linear Ub chains.

2) Figure 4: NEMO NOAZ-OPTN and NOAZ-ABIN2 did not fully rescue NF-kB activation in JM4.5.2 cells, but they did in NEMO-/- MEFs. Could this be due to a difference in protein expression levels? A western blot of the lysates should help to evaluate this result.

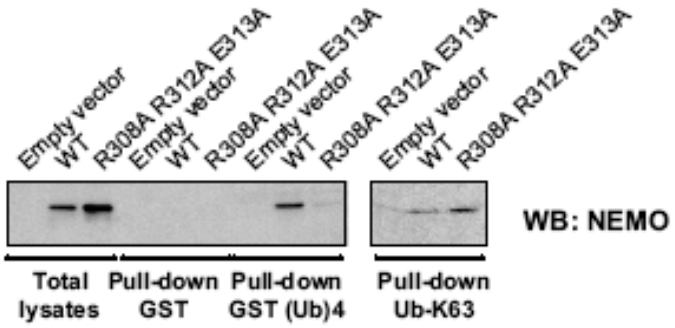
1st Revision - authors' response 04 July 2009

Please find enclosed a revised version of our manuscript entitled “NEMO recognizes K63-linked polyubiquitin chains through a new bipartite ubiquitin binding domain”, modified according to the comments of the 3 reviewers, whom we thank for their constructive criticisms. The 2 important conclusions of our study are : the Zinc Finger (ZF) of NEMO contributes to its specific and high affinity binding to K63-linked chains, but does not contribute to binding to linear chains. Reviewer 1 disagrees with this conclusion, but we now provide evidence that irrespective of the length of the linear chain, the ZF is not involved. The second conclusion is that the affinity of full-llength NEMO for K63 linked chains is as strong if not stronger than for linear chains. This is of course not the case for the isolated NOA, which binds linear chains with a higher affinity than K63-linked chains.

Our point by point response to the comments of the reviewers are as follows:

Reviewer 2: we thank this reviewer for his/her strongly supportive comments. Regarding the expression levels of NEMO in reconstituted cells, we have done the requested controls and included a Western blot of the expression level of the transfected NEMO derivatives in the new figures 2 and 4: see response to point 5 of reviewer 1.

Reviewer 3: we thank this reviewer for his/her strongly supportive comments. Regarding the expression levels of NEMO in reconstituted cells, we have done the requested controls and included a Western blot of the expression level of the transfected NEMO derivatives in the new figures 2 and 4: see response to point 5 of reviewer 1. Regarding the Rahighi’s triple mutant that affects binding to linear but not to K63-linked polyubiquitin chains, we have performed pulldowns of linear and K63-linked tetraubiquitin using extracts from cells transfected with wt and triple mutant full-length NEMO. The results (figure below) confirm that mutant NEMO binds linear chains with a strongly reduced affinity, while it binds K63 chains with a similar affinity as wt NEMO. Deletion of the ZF reduces the affinity of wt and mutant NEMO for K63 chains to an almost undetectable level (similar to what is seen in figure 3, lane 5), while their affinity for linear chains remains the same, as expected (not shown). Clearly, further studies are required to determine whether NEMO binding to linear chains is of physiological relevance, and to compare it to binding to K63-linked chains. In particular, while the triple mutation studied by Rahighi & al. seems to point to the physiological importance of binding to linear chains, the strong in vivo consequences of mutations or deletion of the ZF point to an important role of binding to K63-linked chains.



The R308A R312A E313A mutant of NEMO is defective in its ability to bind linear but not K63-linked ubiquitin chains. Wild-type murine NEMO and the R308A R312A E313A mutant were expressed in NEMO-deficient MEFs. Lysates were prepared and used for pull-down assays with linear (GST-(Ub)4) or K63-linked polyubiquitin chains (2-6 ubiquitin-long chains synthesized in vitro and coupled to Sepharose according to Ea et al. Mol. Cell. 2006, 22(2):245-57). GST was used as a control. Pull-downed material was visualized by western blotting using an anti-NEMO antibody.

Reviewer 1: we have addressed the points raised by the reviewer by performing a series of additional experiments.

- point 1: «the reviewer cannot agree with the authors' opinion that ZF is coordinately involved in K63 chain, but not in K48 or linear, recognition».

As a preamble, we do not claim that binding to K48 chains is not dependent on the ZF, in fact we show that it actually is (Table 2), although the effect is modest (see response to point 3). The statement of the reviewer is partially based on the following argument: we are able to see the effect of the ZF on binding to K63-linked chains only because we use Ub3-7 (therefore favoring binding to the long 6- and 7-mer), but we don't see it with linear tetraUb because it is too short to accomodate both NOA and ZF. This is an important point, and we have performed the following experiments to address it:

1) as a control we have repeated the pulldown experiments using K63-linked chains of length 5, 6 and 8. As can be seen in the new figure S2, panel A, neither the apparent affinity nor the effect of the ZF seems to vary significantly with the chain length. Such a result was already suggested by the following observation : in the pulldown exps using the 3-7 mixture of K63 chains (figure 3), there was no overrepresentation of the six- or seven-mer in the pulldown when compared with the input. Only (Ub)3 seemed to be underepresented.

2) we have then performed a pulldown using either linear tetraUb or linear nonaUb chains with NOA and NOAZ. As shown in panel B of figure S2, deletion of the ZF does not seem to interfere with binding to the 9-mer. To confirm this result we have measured the actual affinity of NOA and NOAZ for the linear nonaUb chain : as can be seen in the new Table 2, the affinity of NOA and NOAZ for the linear nonaUb chain is identical to the affinity for the linear tetramer, confirming that irrrespective of the length of the linear chain, the ZF is not involved in the binding of NEMO, or at least if the ZF binds the polyUb chain, it does not modify the affinity of the NOA. As discussed in the text, the reason is probably that the affinity of the NOA for linear Ub chains is so high that the presence of the ZF does not make a difference, which is not the case with K63-linked chains. These new data are now presented in the Results and Supplementary Information sections. Regarding K63-diUb, we indeed do not see any difference with or without the ZF (not shown), suggesting that the K63 chain needs to be longer than a trimer to accomodate the 2 UBDs.

We also want to stress the fact that the difference in affinity of NOA for K63-linked vs linear chains, which is ~100 fold for tandem diubiquitin in the single paper where it has been measured (Lo & al., 2009) decreases with increasing chain length, and is only ~10 fold for tetramer and longer chains (new Table 2), a decrease which is predicted and discussed by Rahighi & al. on page 7 of their manuscript.

- another argument of the reviewer against our model is : *«It is unclear why there would be a specificity for K63 polyubiquitin chains».* The affinity measurements clearly show that there is a specificity for K63 vs K48, and that the affinity of the NOAZ for K63-linked chains is even higher than that of the NOA or NOAZ for linear chains. Obviously the determination of the structure of the NOAZ in complex with K63-linked chains will be necessary to understand why it binds K48 chains less efficiently. It is important to remember that before the structure was solved (Rahighi & al., 2009), there was no particular reason to believe that the NOA would bind linear chains more efficiently than K63-linked chains, especially after seeing the comparative structure of linear and K63 chains (Komander & al., 2009). Similarly, and more relevant to the bipartite nature of the NOAZ domain, only the determination of the structure allowed to understand why the bipartite UBD of RAP80 shows specificity for K63 vs K48 chains (Sims & Cohen, Mol Cell, 2009 33, 775-783; Sato & al., EMBO J, 10.1038/emboj.2009.160). It could be speculated that simultaneous binding of NOA and ZF to K48-linked chains is less efficient than binding to K63-

linked chains, or that it is not cooperative, because of the strong difference between the structures of these two types of chains. Regarding binding of NEMO to linear chains, it is unclear whether the ZF binds simultaneously with the NOA, but if it does, the effect is obviously not synergistic, possibly because the affinity of the isolated NOA for this type of chain is already very high.

- point 2: *«The authors should examine affinity of linear polyubiquitin binding to ZF (not NOAZ)»* We have measured this affinity, and present the results below: they demonstrate that the ZF shows no specificity for either linear, K63 or K48 chains.

NEMO ZF

	Kd (chain concentration) µM
K63-tetra-Ub	**17+/-2**
K48-tetra-Ub	**18+/-0.5**
linear tetraUb	**27+/-3**

- point 3: *«The authors should also explain binding of K48 polyubiquitin chains to NOA and NOAZ in the light of their potential function in NEMO»* We are not totally sure to understand what the reviewer means. It is clear that both NOA and NOAZ also bind K48 chains in vitro, and there is indeed a modest gain of affinity when the ZF is present. Indeed all chain-specific UBDs described so far only show a modest specificity factor. In fact NEMO is rather unique with a 30-50 fold specificity factor for the NOAZ. The real question is whether NEMO binds or not K48 chains in vivo, and whether this has functional consequences, but it is almost impossible to answer this question with the tools currently available. This is indeed a recurrent background question in the field: is Ub chain preference important for the in vivo function of Ub-binding receptors (see Kim and Rao, Sci. STKE 2006 (330), pe18. [DOI: 10.1126/stke.3302006pe18]). In the case of mutants that abolish the activity of the ZF, the affinity for K48 and K63 of the remaining NOA is similar, indicating that under these conditions NEMO might bind to K48 or K63 chains with an equal probability. However this does not help as these molecules are essentially poorly active or inactive. It may be possible to manipulate the composite UBD to make it specific for K48 chains, and then test whether it complements NEMO-deficient cells. We think this is however outside of the scope of this manuscript.

- point 4: *«The authors claim that the linker (which varies among NEMO, Optineurin and ABIN2) is dispensable. More experiments need to be done to support this statement»* We do not claim that the linker is completely dispensable in an *in vivo* system, for example in an animal (especially as the linker region contains a probable binding site for CylD, as well as *in vivo* phosphorylation sites). However we claim that NEMO deleted of its linker region binds K63 and linear chains with affinities similar to WT NEMO, and that it can reconstitute NEMO-deficient cells as efficiently as WT NEMO. So within the limits of these relatively crude assays, it seems to be dispensable. Of course a knock-in of the deletion in the mouse might result in more subtle differences. We have rephrased the text in order to clarify this issue.

- point 5: *«Functional studies presented in this manuscript are very limited and poorly performed. For example, in experiments using NEMO deficient cells, the expression level of NEMO mutants is very important. The expression level of NEMO and its mutants must be at the comparable level of endogenous level (namely the expression level of parent cells or other MEFs). When overexpressed we cannot see effects of subtle changes provoked by mutations of NEMO»* This point has also been raised by the 2 other reviewers. We have now performed Western blotting for NEMO in reconstituted NEMO-deficient MEFs and in JM4.5.2 cells, in parallel with luciferase measurements. The results are presented in the new figures 2 and 4 (and the original figure S1 has been removed). These new data show that the expression levels of the various mutants are similar if not identical to that of wt NEMO. The only construct that shows a slightly higher expression is the NEMO ZF-WRNIP1 (D37A), but transfection with this mutant has been repeated multiple times using different DNA concentrations, and it has consistently exhibited a much weaker activity than NEMO ZF-WRNIP1.

point 6: *«Tables 1 and 2: Authors should use the same constructs in all the experiments in the manuscript: isolated ZFs, CC2-LZ, NOAZ various chimeras etc. for the sake of intrinsic consistency. It would also be beneficial to their story if they performed the fluorescence spectroscopic analyses with K63, K48 and linear- diUb chains in order to confirm or challenge the recently published studies (Lo et al., 2009, Komander et al., 2009 and Rahighi et al., 2009)»*
Regarding the different types of chains which are used in this manuscript (this only concerns the NOA fragments, as for the ZF, we only use chemically synthesized peptides, which are the same throughout the manuscript), we agree with the reviewer and have measured the affinity of 3 types of recombinant NEMO fragments for GST-(linear tetraubiquitin). These results are shown below (measured by fluorescence polarization) and indicate that all fragments bind polyubiquitin with a similar affinity:

construct	K_D (μM)
aa 253-336 (NOA, mu)	2.8 ± 0.5
aa 215-362 (NOA, hu)	2.0 ± 0.5
aa 241-386 (NOA, mu)	3 ± 0.4
aa 241-412 (NOAZ, mu)	3.9 ± 0.5

This very similar affinity (for linear Ub) found using 3 different peptides representing the CC2-LZ (and one NOAZ as a control) suggests that the exact coordinates of the CC2-LZ do not matter too much as long as it includes the CC2 and the LZ domains. Indeed it is unclear where exactly the CC2 starts, as the structure determined by Lo & al. and Rahighi & al. only starts at residue 260. Similar results have been obtained for K63-linked chains.

Regarding measurement of the affinity for K63 or K48 diubiquitins, and comparison with the data of Lo & al. and Rahighi & al., we feel that in the context of our manuscript, these measurements are not very useful as these short chains do not allow simultaneous binding of NOA and ZF, thus bind NOAZ similar to NOA, and therefore cannot be used to test our model.
In addition we want to insist on the following points:

1) Lo & al. find affinities of NEMO CC2-LZ (aa 251-350) of 1.4 and 141 μM for linear and K63 diUb, respectively, using ITC, while Rahighi & al. measure an affinity of 1.6 μM of CC2-LZ (aa 250-339) for linear diubiquin by surface plasmon resonance (this technique did not allow to measure the affinity of K63 or K48 chains). Our study is far more extensive as it measures affinity of NOA and NOAZ for several lengths of linear, K63 and K48 chains. Using fluorescence polarization as well as fluorescence competition, we measure an affinity of CC2-LZ (aa 253-336) of 2.2-2.8 μM for linear tetraubiquitin, which is remarkably close to the value obtained by Lo & al and Rahighi & al. for diubiquitin. Our measurement of the affinity of the NOA for K63 chains gives a value of 25 μM, which is higher than the affinity observed by Lo & al. This is probably due, as mentioned above and discussed in point 1, to the longer Ub chain used for the measurement (tetra- vs diubiquitin). Indeed the difference in affinity of the NOA for linear vs K63-linked chains which is close to 100-fold (for diubiquitin) in the Lo & al. paper decreases with increasing chain length (see new Table 2, where the difference for a tetra-Ub is ~10 fold); such a decrease in the ratio has been postulated in the discussion of Rahighi & al., 2009, and interpreted as a consequence of an increased affinity of NOA for K63-linked chains when the chain length increases from 2 to 4.

2) the point of our paper is not to determine whether the NOA binds linear polyUb 100-fold (for diubiquitin) or 10-fold (for tetraubiquitin) better than K63-linked polyUb (although NEMO is more likely to meet long chains than diubiquitin in the cell), but to demonstrate the involvement of the ZF in binding to K63-linked chains but not to linear chains, and also incidently that full-length NEMO is unlikely to bind linear chains with more affinity than K63-linked chains. In that sense the data in the Lo & al. and Rahighi & al. papers cannot be compared to ours.

- point 7*: «Authors should use WT forms of proteins to create chimeras in their luciferase assays as controls, perform appropriate quantification methods and statistical analyses when making conclusions and provide transfection controls in the same cell types used for the study»* We apologize but it is not entirely clear to us what the reviewer means by: Authors should use WT forms of proteins to create chimeras in their luciferase assays as controls. We do use wt NEMO to create the ZF and NOAZ swaps, and the transfection of wt NEMO is included in the experiments. In addition using wt Optn or ABIN2 to complement NEMO-deficient cells will not work (we have demonstrated this already for Optn in Schwamborn & al. J Biol Chem, 2000, 275, 22780-22789). Then the transfections have been repeated at least 9 fold, and the error bars are indicated. Finally, as discussed in point 5, the expression levels of the constructs used are now included in figures 2 and 4.

- point 8 *: «Data should be explicitly shown for the complementation experiments of F312W mutation (mentioned in page 8) since the F312A mutation has been shown to affect ubiqitin binding by several groups»* This is an important point, and the actual data have now been included as new figure S4, and demonstrate that F312W complements NEMO-deficient cells a well as wt NEMO, contrary to the situation observed for F312A (the Western blot confirms that the expression levels of wt NEMO and F312W are identical).

We hope this answers the various criticisms raised by the reviewers, and that our manuscript will now be considered suitable for publication in EMBO Journal.

2nd Editorial Decision 16 July 2009

Thank you for submitting your revised manuscript to The EMBO Journal. As the editor handling this manuscript is away this week and next, I am stepping in to avoid delays.

The revised version was sent back to the original refs 1 and 3 and their comments are shown below. Referee # 1 still has some issues as you can see. To ensure that the final manuscript is as solid and convincing as possible, it would be good if you could address these points by data or by commentary before we go forward. It does seem to me that, given the importance of the specificity for K63 chains, making sure the titration curves in Figure S3 are good and clear (the K63 NOAZ low data points look perhaps a bit odd - biphasic?) is worthwhile. Note also if some data are key they may also be better appreciated in a main Figure than in a supplemental one, but I leave that decision to you.

When you send us your revision, please include a cover letter with an itemised list of all changes made as well as your answers in response to the referees.

Thank you again for the opportunity to consider your work for publication. We look forward to your final revised manuscript.

Yours sincerely,

Executive Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

One important conclusion of this manuscript is the necessity of K63-linked polyubiquitins to bind to NEMO ZF domain and hence activate NF- B and also defective NF- B activation by NEMO delta ZF (Figure 2A). This would be an important and very much needed contribution to the field if the

authors can clearly demonstrate this point. In the revised manuscript the authors have not been able to answer several critical points. New experiments raise several critical issues regarding the technical performance that were not present in the first version. Additional experiments are needed to strengthen their conclusions:

Major points:

1. There is no direct evidence indicating that it is indeed K63-linked chains that activate NEMO, hence the NF-κB signaling. There is a lot of circumstantial evidence for the significance of K63 chain binding, but no single experiment showing the direct involvement of the K63 chain in the NF-B activation. Except for the 10 times stronger binding towards K63 over linear chains, which should be better evidenced (see below), there is no reason to exclude the involvement of other type of polyubiquitin chains, like linear polyubiquitin chains, in the NF-κB activation assays shown in Figures 2 and 4.

2. Figures 2A and 4A show that any ZFs attached to NEMO is functional in combination with NOA for recognizing K63-linked Ub chains and for NF-κB activation, how can it be explained that NEMO ZF-OPTN (or NEMO NOAZ OPTN) and NEMO ZF-ABIN2 (or NEMO NOAZ-ABIN2) chimeras do not function as good as NEMO wt in regulation of NF-κB activation? If K63-chain recognition is the only critical point, these results cannot be explained since these chimeras bind K63 chains as potent as NEMO wt or even more.

3. It is not clear why the affinities of tetra and nona linear ubiquitin chains towards NEMO do not differ between CC2-LZ (2.2 μM for tetra and 2.0 μM for nona-Ub) and NOAZ (1.7 μM for tetra and 1.9 μM for nona-Ub). The authors argue that NOA's affinity for linear ubiquitin is already strong and the ZF domain probably does not contribute to the affinity. This does not really make sense since NEMO ZF does not have preference towards linear, K48, or K63 chains, and Kd values are on the order of 17 to 27 μM (these numbers should be included in Table 1).

4. The authors claim that NOAZ (NOA+ZF) binds K63-linked polyUB more strongly than K48 (32 times) and linear (about 10 times) chains as shown in Table 2. In the left panel of Supplementary Figure S3, the first part of the plots for K63-linked chains, which resulted in Kd value of 0.3 μM against NOAZ, is too crowded to see the validity of the fitting. Perhaps, a blowup figure in inset might be helpful here. For better comparison, Supplementary Figure S3 should really contain a panel for linear ubiquitin (without GST tag) to show that there is indeed no change in affinity between NOA and NOAZ.

5. Figure in the rebuttal letter.
"The R308A R312A E313A mutant of NEMO is defective in its ability to bind linear but not K63-linked ubiquitin chains." could be misleading. First the residue number is wrong; R309A not R308A. Second, they used again tetra-ubiquitin chain, which may not be long enough. For K63 chain, they used a range of oligomers from 3 to 6. It may so happen that the R309A R312A E313A mutant would bind linear ubiquitin chain of sufficient length (there is no reason why ZF should NOT bind linear UB in this context).

To be corrected:
1. Table 2 and Figure S3. The KD values written in the left panel (0.3 μM and 35 μM) do not correspond to those listed in Table 2 (0.22 μM and 25 μM), while those in the right panel (9.5 μM and 45 μM) do.

2. The second sentence of the Abstract, "A small domain called NOA/UBAN has been suggested to be responsible for this property (recognition of K63-linked polyubiquitin chains)" is rather problematic. It does not reflect the recent literature (D. Komander et al., EMBO Reports; Rahighi et al. Cell; Hao et al., Mol Cell), nor consistent with the last sentence.

3. the authors did not fully answer the question #5 from Reviewer 1. The important point is to compare the protein expression levels of endogenous NEMO and reconstituted NEMO that are not shown in the revised manuscript.

4. There are some sentences need to be rephrased;

page10, first sentence in a paragraph 'different requirements for .....'; the authors wrote 'NEMO might be ubiquitinated... '. this should be rephrased to 'NEMO is ubiquitinated' based on the clear evidences shown in a published paper by Tokunaga et al.

similar with a sentence in page 16, lane 11, the authors wrote 'NEMO might be ubiquitinated...'. this should be rephrased.

5. Figure 3 needs to be repeated with purified proteins since with the data shown here, it maybe possible that these Ub chains bind each of the proteins directly. However, it is not at all clear why one uses IPed sample for such experiments.

Referee #3 (Remarks to the Author):

This revision has addressed my concerns. I think this is an important and well-executed study that should be published in EMBO Journal. The study provides new insights into the mechanism of NEMO functions, and will have significant impact in the NF-kB and ubiquitin fields.

2nd Revision - authors' response 22 July 2009

Please find enclosed a re-revised version of our manuscript entitled “NEMO recognizes K63-linked polyubiquitin chains through a new bipartite ubiquitin binding domain”, modified according to the comments of reviewer 1. The manuscript has been modified to answer points 4 and 5, as well as the corrections requested at the end of the review.

Our point by point response to the comments of reviewer 1 is as follows:

- point 1 : *«1. There is no direct evidence indicating that it is indeed K63-linked chains that activate NEMO, hence the NF- κB signaling. There is a lot of circumstantial evidence for the significance of K63 chain binding, but no single experiment showing the direct involvement of the K63 chain in the NF-κB activation»* This is an important issue, but certainly not the one we chose to address in our manuscript. Determining whether binding to K63-linked chains and/or to linear chains is important for NF-κB activation is out of the focus of this manuscript, and will certainly involve a large amount of additional work. The only thing we say is that, irrespective of the role of NEMO binding to either type of chain, the requirements for binding are different. We include a few words in the discussion to emphasize the fact that data in the literature suggest that both bindings are important, but this is not the subject of our study. We certainly do not want to imply that binding to linear chains is irrelevant, and nowhere in the manuscript do we suggest anything like that.

- point 2 : *«Figures 2A and 4A show that any ZFs attached to NEMO is functional in combination with NOA for recognizing K63-linked Ub chains and for NF-κB activation, how can it be explained that NEMO ZF-OPTN (or NEMO NOAZ OPTN) and NEMO ZF-ABIN2 (or NEMO NOAZ-ABIN2) chimeras do not function as good as NEMO wt in regulation of NF-κB activation? If K63-chain recognition is the only critical point, these results cannot be explained since these chimeras bind K63 chains as potent as NEMO wt or even more»*

Again we do not claim that K63-chain recognition is the only critical point, we only say it is important, and analyse the requirements for this recognition.

Pulldown experiments are not perfectly quantitative, unlike luciferase activities in transactivation experiments. Therefore is not unexpected that the percentage of NF-κB activation does not totally match the pulldown activity. However it can be noted in figure 3 that the swaps with the ZF of OPTN and ABIN2 bind slightly less well K63 chains that does wt NEMO, something that can be correlated with the sightly lower complementation activity seen in figure 2 (60% of wt). It must be kept in mind that these are chimeric molecules, and the possibility exists that the fusion somehow interferes with the perfect structure of the molecule. However, at least qualitatively, the correlation is very good. Regarding the higher binding activity of the swap with the entire ABIN2 NOAZ

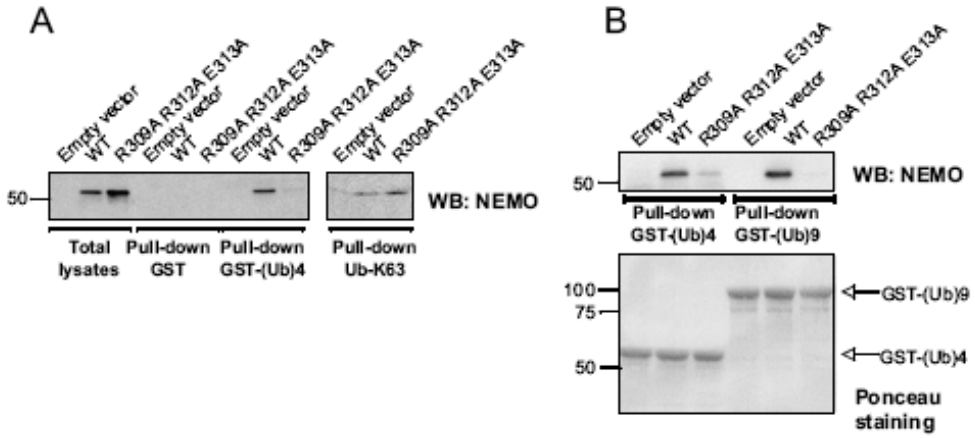
domain, it may be a consequence of the fact that NOA and ZF derived from the same molecule have co-evolved to be better binders than a chimera with a different ZF. In addition, we think that an important result which supports our hypothesis is the difference observed between the swap with wt WNRIP1 ZF and with the mutant of this ZF that is defective in binding to ubiquitin.
Finally it must be kept in mind that activation is not identical to binding, eventhough the 2 are correlated. Activation of the IKKs, that somehow follows binding to polyubiquitin, is likely to require a conformational change in the N-terminal region of NEMO (that binds the kinases) that probably extends throughout the molecule, and therefore the behavior of a chimera might not be exactly identical to that of the wt molecule.

- point 3 : *«It is not clear why the affinities of tetra and nona linear ubiquitin chains towards NEMO do not differ between CC2-LZ (2.2 for tetra and 2.0 μM for nona-Ub) and NOAZ (1.7 μM for tetra and 1.9 μM for nona-Ub); The authors argue that NOA's affinity for linear ubiquitin is already strong and the ZF domain probably does not contribute to the affinity. This does not really make sense since NEMO ZF does not have preference towards linear, K48, or K63 chains, and Kd values are on the order of 17 to 27 μM (these numbers should be included in Table 1)»*
We now have included the numbers in Table 1. Contrary to the opinion of the reviewer, we think the argument makes sense: the affinity of the ZF for for K63 or K48 chains is higher than that of NOA, therefore, following our speculation, it can be expected that the presence of the ZF will improve the affinity of the NOA when both are associated; of course the synergy is weaker for K48 chains, but this is not unexpected based on the very different structures of K48 and K63 chains. Then the affinity of the ZF for polyubiquitin is lower than that of NOA for linear chains, and therefore it is not totally surprizing that the presence of the ZF does not improve the NOA's affinity. This is of course speculative, but we feel it makes sense. Again determination of the structure of NOAZ associated with linear or K63 chains will be necessary to resolve the issue.

- point 4: *«The authors claim that NOAZ (NOA+ZF) binds K63-linked polyUB more strongly than K48 (32 times) and linear (about 10 times) chains as shown in Table 2. In the left panel of Supplementary Figure S3, the first part of the plots for K63-linked chains, which resulted in Kd value of 0.3 M against NOAZ, is too crowded to see the validity of the fitting. Perhaps, a blowup figure in inset might be helpful here. For better comparison, Supplementary Figure S3 should really contain a panel for linear ubiquitin (without GST tag) to show that there is indeed no change in affinity between NOA and NOAZ».*
Regarding the validity of the fitting with K63-linked (3-7) chains, higher accuracy in estimating the Kd is generally obtained if one analyses the entire fluorescence titration curve rather the first region of the binding curve (see Bujalowski et al. 1996 Biochemistry 35, 2117-2128). In particular, the region near the plateau value in Figure S3 is more informative that the first region because it shows little curvature, indicating a good affinity. To clarify this point and for better comparison between linear and K63 chains, we now present in Supplementary Figure S3 four additional binding curves of NOA and NOAZ binding to linear nona-ubiquitin and to K63 tetra-ubiquitin.

- point 5 : *«The R308A R312A E313A mutant of NEMO is defective in its ability to bind linear but not K63-linked ubiquitin chains.» could be misleading. First the residue number is wrong; R309A not R308A. Second, they used again tetra-ubiquitin chain, which may not be long enough. For K63 chain, they used a range of oligomers from 3 to 6. It may so happen that the R309A R312A E313A mutant would bind linear ubiquitin chain of sufficient length (there is no reason why ZF should NOT bind linear UB in this context)»*
We now have convicingly demonstrated that NOAZ binds tetra- or nona-ubiquitin with a similar affinity, and with an affinity similar to that of NOA. Therefore it would be surprizing that the same is not correct for the triple mutant. However there is a remote possibility that the presence of the ZF might help a crippled NOA to bind linear polyubiquitin, if the chain is long enough. To answer this point we have done pulldown experiments with WT NEMO and R309A R312A E313 triple mutant and nona-ubiquitin. The results presented below indicate that 1) WT NEMO binds equally well linear tetra- and nona-ubiquitin and 2) the triple mutant is still ineffective at binding linear nonaubiquitin, similar to the situation observed with tetra-ubiquitin. In fact the answer to this question can also be found in the Rahighi & al. paper : the authors demonstrate that the triple mutant NOA is defective in binding linear tetraubiquitin, and also that triple mutant full-length NEMO is defective in complementing NEMO-deficient cells, indicating that the simultaneous presence of

long chains (likely to be present in cells) and of the ZF is not sufficient to compensate for the triple mutation.



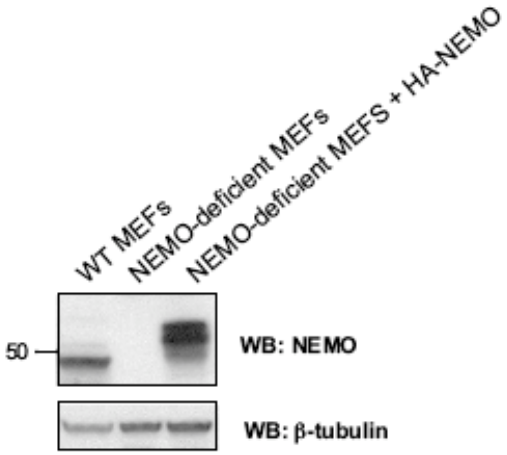
The R309A R312A E313A mutant of NEMO is defective in its ability to bind linear but not K63-linked polyubiquitin chains. A) Wild-type mouse NEMO and the R309A R312A E313A NEMO mutant were expressed in NEMO deficient MEF cells. Lysates were prepared and used for pull-down assays with linear (GST-(Ub)4) and K63-linked ubiquitin chains (2-6 ubiquitin-long K63 chains synthesized in vitro and coupled to sepharose according to Ea et al. Mol. Cell. 2006, 22(2):245-57). GST was used as control. Pull-down material was visualized by western blotting using an anti-NEMO antibody. B) The lysates presented in A) were used for pull-down assays with linear GST-(Ub)4 and GST-(Ub)9. Pull-downed material was visualized by western blotting using an anti-NEMO antibody. GST-fusion proteins were detected by Ponceau-S staining.

Regarding the last point (there is no reason why ZF should NOT bind linear UB in this context), this is correct, and only the structure will tell whether the ZF binds or not. However, even if the ZF binds linear chains, it does not synergize with the NOA, probably for reasons which are discussed in the answer to point 3 (see above).

Points to be corrected:
- 1: *«Table 2 and Figure S3. The KD values written in the left panel (0.3 µM and 35 µM) do not correspond to those listed in Table 2 (0.22 M and 25 M), while those in the right panel (9.5 µM and 45 µM) do»*

- There is no error in the Kd values shown in Table 2 and Figure S3. The left panel is for K63 linked (3-7) chains (NOA: 35 µM and NOAZ: 0.3 µM) and the right panel for K48 linked (3-7) chains (NOA: 45 µM and NOAZ: 9.5 µM). These values correspond to those listed in Table 2. As mentioned above, a new Figure S3 has been included and further explained to avoid any confusion.
- 2 : we have modified the sentence in the abstract.
- 3 : the reviewer wants us to compare the level of expression of NEMO in wt cells vs NEMOdeficient cells transfected with the various NEMO mutants. Doing this is in fact not exactly the right experiment, as the level of NEMO observed in the transiently transfected cells is the result of NEMO expression in the subset of the cells which have been transfected. In fact, in papers addressing similar questions, the mutants of a protein of interest are usually tested in such a way that the level of expression is identical for all mutants, which is what we have done. Another way of doing the experiment would be to establish stable clones for each of the mutants; however this is a lengthy process which will require more than 2 months, and which will most likely, based on our previous experience with NEMO, to give results which are qualitatively, if not quantitatively, identical to what we observe now. However, to comply with this request, within the limits of the transient transfection procedure, we present below a blot showing the level of expression of endogenous NEMO compared with MEFs transfected with wt NEMO. We have also determined the efficiency of transfection to be more than 70% (using GFP), and correcting for this percentage, we conclude that the level of expression of NEMO in transfected cells is within the same range as

the endogenous level of NEMO in wt MEFs (taking into account the loading control of beta-tubulin).



Comparison of the expression level of endogenous versus transfected NEMO. WT MEFs, NEMO-deficient MEFs and NEMO-deficient MEFs transiently transfected with HA-NEMO (in the same conditions as figure 4) were analysed for NEMO expression using an anti-NEMO antibody. ß-tubulin was used as a loading control.

- 4 : we have modified the sentences accordingly.
- 5 : we disagree with this criticism. In fact we are not sure of what the reviewer means by *: «Figure 3 needs to be repeated with purified proteins since with the data shown here, it maybe possible that these Ub chains bind each of the proteins directly»*, but maybe the reviewer means these Ub chains bind each of the proteins indirectly. In fact we had already repeated the pulldowns with some, although not all, of the purified recombinant proteins (figure S1), and this clearly demonstrates that the binding is direct; therefore there is no particular reason to believe that this would be different for the other proteins used in figure 3.

We hope this answers the various criticisms raised by the reviewer, and that our manuscript will now be considered suitable for publication in EMBO Journal.

