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c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signaling

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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 10 August 2010

Thank you again for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, they all consider your results as a first implication of K11-linked ubiquitin chains in TNF signaling interesting and potentially important and thus in principle suited for publication in The EMBO Journal. Nevertheless, they also all raise a number of major as well as more specific issues with the manuscript in its current form. While I do not feel that all of them will necessarily have to be addressed through further experiments to warrant successful further consideration, one point raised especially by referees 2 and 3 will however be very important, which is to provide stronger and more direct evidence for the actual involvement/importance of K11-linked chains in TNF/NF-kB signaling. On the other hand, some of the more specific points (such as extending certain experiments also to homologs/paralogs) that would round up the analysis may not be strictly required, unless of course unraveling potential redundancies might help in uncovering the physiological significance of the TNF-induced K11 chains as mentioned above.

Therefore, should you be able to improve this main point, as well as to adequately address the other main issues, then we should be able to consider a revised version of the manuscript further for publication. I should however point out that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various experimental and editorial points raised at this stage. In a revised version, please also briefly indicate the individual author contributions, either in the acknowledgements section or in an adjacent separate section, as we are attempting to adopt this as a common policy now. Finally, when preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website:

http://www.nature.com/emboj/about/process.html).

Should you need feedback on any issue regarding this revision and its requirements, please do not hesitate to contact me for further discussion. Thank you for the opportunity to consider your work for publication - I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript of Dynek et al. examine the UbcH5-c-IAP1 E2-E3 complex in placing K11 ubiquitin chains onto the E3 itself as well as its substrates NIK and RIP1. Starting with yeast two-hybrid screens to identify novel E2 interaction partners for the IAP E3s, the authors performed in vitro and in vivo ubiquitination reactions to elucidate K11 chain formation. Furthermore, Dynek et al. evaluate K11 chain conjugation in activated NF-kappaB pathways and end their manuscript with analyzing the binding of NEMO to K11-linked chains.

K11 chains were recently described to play a role in progression through mitosis, catalyzed by the APC/C E3 ligase complex in combination with UbcH10 and UBE2S (Jin et al., Cell, 2008; Xu et al., Cell, 2009; Wu et al., PNAS, 2010). It has been shown that substrates marked with K11 chains are targeted to the 26S proteasome for degradation (Xu et al., Cell, 2009; Wu et al., PNAS, 2010). These findings, together with recent reports emphasizing K11 chain structures (Matsumoto et al., Mol Cell, 2010; Bremm et al., NSMB, 2010) and recent insights in differential ubiquitin chains and their binders in NFkappaB signaling, illustrate the interest, timing and relevance of this manuscript. The manuscript will be further improved by addressing the following questions:

Major points:
1. The authors only observed in vitro UBE2S activity when the E3 was pre-incubated with a sub-optimal amount of UbcH5B, but did not show that the catalytic activity of UBE2S is causing this effect. Furthermore, it remains unclear if the ubiquitin signals are derived from c-IAP1 autoubiquitination or unanchored ubiquitin chains (as known for UbcH5s). Dynek et al. should also include an UBE2S-binding deficient IAP RING domain and an E2-binding deficient mutant. Additionally, they could have determined the type of ubiquitin linkages in the first and second step reactions both with UbcH5A and UBE2S, using mass spectrometry.

2. In Figure 3, Dynek et al. stimulated cells for different time-points with CD40L, combined with knock-down of individual E2 enzymes and c-IAP1 stability as read-out. Here, the authors did not show that stimulation was effective by a read-out other than c-IAP1 stability. Also, they must show how other IAPs (interacting positively with UbcH5B in Y2H) behave, since IAPs are often found in complex with each other and could have redundant functions. Additionally, no explanation is given for the absence of any effect on c-IAP1 stability upon UBE2S knockdown.

3. In addition, NIK ubiquitination was evaluated after over-expression of c-IAP1 and -2 in combination with E2 siRNA. Here, the authors should use c-IAP1/2 mutants in the E2 interaction interface/dimerization mutants next to wild-type E3s to strengthen their conclusions. As mentioned before, an explanation why a knockdown of UBE2S does not lead to NIK stabilization is also lacking.

4. The authors investigated the E3 activity of c-IAP1 towards itself and RIP1, using over expressed RIP1, c-IAP1 and several ubiquitin mutants, followed by IPs. Again, any control, like a c-IAP1 dimerization or E2-binding mutant, is missing, just like the use of K11R, K48R and K63R mutants. The in vitro ubiquitination reaction in Figure 4B should be done also with K11R ubiquitin and the

effect of UBE2S is not determined.

5. Furthermore, an in vitro approach was pursued, combined with MS analysis to study the type of chains generated by c-IAP1 and UbcH5A. Here, the authors should have quantified all potential types of ubiquitin chains, since UbcH5A is capable of creating mixed chains. Additionally, the concluding sentence at page 9 could only have been done if a parallel experiment with K11R and determination of the ubiquitin-acceptor lysine on RIP1 was performed.

6. Dynek et al. used TNFalpha stimulation to investigate the in vivo relevance of c-IAP1 in K11 chain synthesis during signaling. c-IAP1 knock out MEFs, reconstituted with wt or mutant c-IAP1 should give rise to more clearness of the direct role of c-IAP1 and K11 in signaling. The authors must explain why in Figure 5D, knock down of UbcH13 and UBE2S yield more RIP-1 ubiquitination in combination with K11R mutants and MS analysis to demonstrate K11 involvement.

Minor points:
1. The authors tested four different IAP RING-finger domains against a panel of 30 human E2 prey constructs and detected selective E2-E3 interactions. Later on, E2 interface mutants in the RINGs were generated, displaying altered E2 interactions. The authors did not show how these RING mutants interact with all other E2s within their panel. Additionally, the sequence alignment in Figure 1C showed a very conserved RING domain for c-IAP1, c-IAP2 and ML-IAP, but the authors do not discuss why these conserved RINGs display deviant E2 interaction patterns. In addition, the authors did not include UbcH10 in their screening.
3. The authors should mention that some E2 enzymes, like RAD6B and UBE2Q2, have close homologous that could provide redundant functions.

Referee #2 (Remarks to the Author):

The ubiquitin conjugation system has shown to play crucial roles in NF-kappaB activation and various types of polyubiquitin chains including K48, K63, and linear polyubiquitin chains, have shown to be involved in the pathway. In this manuscript, the authors demonstrated the involvement of another polyubiquitin chain, the K11 chain in the NF-kappaB activation pathway using newly developed anti-K11 chain specific antibody. The authors screened E2 partners of IAP RING domains using a directed yeast two-hybrid system, and identified some novel and known E2s. Among these E2s, they focused on UbcH5s (UbcH5a, UbcH5b, and UbcH5c) as E2 partners for cIAP1 and c-IAP2. Using siRNA-mediated knockdown of UbcH5s, the authors indicated that UbcH5s are involved in degradation of c-IAP1, c-IAP2, and NIK, which are the known targets of c-IAPs. The authors also showed that c-IAP1 and UbcH5a promote K11-linked polyubiquitination of RIP1 besides K48 and K63 chains. Moreover, the authors showed that the ubiquitin binding motif of NEMO, the regulatory subunits of IkB kinase (IKK) complex, can bind to K11 di-ubiquitin in the almost same affinity as K48 and K63 di-ubiquitin and that NEMO can bind to RIP1 conjugated with K63 and/or K11 linked chains. Since it has been reported that TNF-alpha-induced IKK activation requires Ubc5s and non-K63 polyubiquitination of RIP1, it is interesting that K11-linked chain may be involved in NF-kappaB activation.
Although the data are interesting, there are a lot of caveats in this manuscript. For example, the authors showed knockdown of UbcH5s can suppress CD40L-induced RIP1 ubiquitination, but they have not shown whether UbcH5s knockdown also suppresses CD40L-induced NF-kappaB activation or not. Therefore, this manuscript cannot be included in the EMBO Journal in the present form. My comments are listed below.

Major comments
1. As pointed above, the authors have not shown that UbcH5s knockdown suppress CD40L-induced NF-kappaB activation or not using their siRNAs for UbcH5s although they showed knockdown of UbcH5s can suppress CD40L-induced RIP1 ubiquitination. Without this result, the reviewer feels that involvement of K11-linked chains in NF-kappaB activation is not sure.
2. K11 chains have shown to function as a degradation signal. The reviewer is curious whether K11 chains conjugated to RIP1 also functions as degradation signals as K48 chains do or not.
3. The authors have shown that c-IAP1 can generate K11-linked chains together with UbcH5a. However, they have not examined whether c-IAP1 can generate K11 chains together with UbcH5b

or UbcH5c. Although the authors have shown that siRNA mediated knockdown of UbcH5s can suppress K11 chain conjugated RIP1 (Figure 5D), they have used mixture of siRNAs targeting UbcH5a, and both UbcH5b and c. Therefore, the reviewer feels it is critical to examine this point to demonstrate the involvement of K11 chains in NF-kappaB activation.
4. In the same line, it has been suggested that c-IAP1 and c-IAP2 play redundant functions. The authors showed that c-IAP1 can generate K11-linked chains in the presence of UbcH5a, but they have not examined whether c-IAP2 can generate K11 chains or not. This is another important point to be addressed to show the involvement of K11 chains in NF-kappaB activation.
5. The authors have shown that knockdown of UbcH5s suppresses degradation of c-IAP1, c-IAP2, and NIK. It might strengthen the authors' observation if introduction of UbcH5a, UbcH5b or UbcH5c, which are resistant for the siRNAs, can overcome suppression of the degradation of c-IAP1, c-IAP2, and NIK induced by the introduction of siRNAs for UbcH5s.
6. In Fig3, the authors indicate that UbcH5s are involved in the degradation of c-IAP protein and its target NIK. However, this conclusion is obtained using CD40-overexpressing HKB11 cells. Is this data reproducible using primary human B cells or B cell lines expressing endogenous CD40? Human B cells can be easily knockdown by lentiviral vector. Furthermore, The reviewer is wondering if c-IAP1 degradation is inhibited by stimulations other than CD40 ligand (ex. BAFF).
7. It has been reported that TNF receptor activation resulted in the recruitment of not only NEMO but also TAB2 in a ubiquitination-dependent manner. It has been suggested that TAB2 binding to K63-linked chain of RIP1 activates TAK1 and IKK. Considering K11-polyubiquitinaiton of RIP1 under CD40L stimulation, it is possible that TAB2 can bind to K11-linked chain as well as NEMO. The reviewer is curious to know whether TAB2 can bind to K11-linked chain by the same method of Fig.6.

Minor comments
1. The authors mentioned in "Introduction" that K63-linked ubiquitin chains might not be essential for TNF-alpha stimulated NF-kappaB activation (Page 3, line 16). The authors showed that RIP1 can be conjugated both K11 and K63 chains by UbcH5s, and the ubiquitin-biding domain of NEMO can bind to both K63 and K11 with almost equal affinity. If K63 chains might not be involved in NF-kappaB activation, it might be also the case with K11 chains. The reviewer recommend to correct the sentence cited above.
2. Fig.1; ML-IAP RING 254 is not V in Fig.1C. Furthermore, there is no sequence of ML-IAP 296 in Fig.1C. Is it correct the sequence number of ML-IAP RING in Fig.1C?
3. It is helpful to understand the meaning of some abbreviations (ex. rxn) if the authors provide full name of the abbreviations.

Referee #3 (Remarks to the Author):

In this manuscript titled Dynek et al demonstrate that c-IAP1 and UbcH5 family of E2 enzyme UbcH5a promote K11-linked ubiquitination of RIP1, an essential molecule of TNF-induced NF- B activation. They show that TNF -induced TNFR1-associated signaling complex includes RIP1 modified with K11-linkages. They infer that this K11 ubiquitination is mediated by c-IAPs because treatment with BV6 resulted in the loss of both c-IAP1 and modified RIP1. The authors demonstrate that ubiquitin-binding domain (UBD) of NEMO, a critical component of IKK complex involved in the activation of canonical NF- B activation, bind in vitro to ubiquitin dimers of varying linkages including K11. Using overexpression system, association between FLAG-NEMO and RIP1 ubiquitinated with K11 linkages was shown. Recruitment of c-IAPs to the TNFR1 and their role in RIP1 polyubiquitination upon TNFa stimulation are known and the authors finding that c-IAP1 promote K11-linked ubiquitination of RIP1 is new and is important. By and large I think the data presented here support the authors' claims however, I'm not convinced that the work presented here goes far enough and I have a few specific comments that I think the authors should address.

1. The authors imply that c-IAP1 linked K11-linked ubiquitin chains are involved in TNF -induced activation of NF- B. Direct evidence for this is needed. Results presented in figure 3 clearly show that upon CD40L stimulation, c-IAP1/UbcH5-mediated ubiquitination events promote degradation of both c-IAP1 and its substrate NIK. How can the authors rule out that in TNF-stimulated cells, RIP1 ubiquitination with K11 linkages does not lead to RIP1 degradation? Binding of NEMO to K11-linked ubiquitin chains alone does not rule out such a possibility, because results presented in figure 6C and earlier reports show that NEMO can associate with K48-linked chains, known to

promote protein degradation. Identification of lysine residues on RIP1 modified by c-IAP1 with K11-linkages and characterization of importance of the lysine residues, by mutation analysis, in NF-B activation would support the authors' claims.

2. The authors conclude on page 11 that: "... these results indicate strongly that K11-linked ubiquitin chains can serve as a molecular signal for recruitment of NEMO and participate in TNF signaling." Direct evidence under physiological conditions for participation of K11 linkages in NF- B or TNF signaling is required. The authors should at least show association between endogenous NEMO and endogenous RIP1 with K11 linkages in TNF-stimulated cells.

3. In order to demonstrate involvement c-IAP1 in K11-linked ubiquitination of RIP1, cells treated with the IAP antagonist BV6 were used. The authors show that treatment with BV6 blocks TNF-induced RIP1 ubiquitination and I B degradation, suggesting inhibition of IKK activation. Interestingly, BV6 has been shown earlier to promote both canonical and non-canonical NF- B activation (Figure 5, Cell. 2007, 131:669-81). Can the authors explain this apparent contradiction with their earlier published data. To exclude non-specific effect of BV6, data from cells deficient in both c-IAPS are needed.

1st Revision - authors' response 02 October 2010

Point-by-Point Response
Re: "c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signaling" by Dynek et al.
We are most grateful to the reviewers for their helpful comments. Our point-by-point response follows. We have performed a number of suggested experiments to address the reviewerís comments. Additionally, we sincerely hope that our explanations, which in many cases is simply a clarification of either our data or of the published literature, will help clarify any misconceptions.

Referee #1 (Remarks to the Author):

The manuscript of Dynek et al. examine the UbcH5-c-IAP1 E2-E3 complex in placing K11 ubiquitin chains onto the E3 itself as well as its substrates NIK and RIP1. Starting with yeast two-hybrid screens to identify novel E2 interaction partners for the IAP E3s, the authors performed in vitro and in vivo ubiquitination reactions to elucidate K11 chain formation. Furthermore, Dynek et al. evaluate K11 chain conjugation in activated NF-kappaB pathways and end their manuscript with analyzing the binding of NEMO to K11-linked chains.
K11 chains were recently described to play a role in progression through mitosis, catalyzed by the APC/C E3 ligase complex in combination with UbcH10 and UBE2S (Jin et al., Cell, 2008; Xu et al., Cell, 2009; Wu et al., PNAS, 2010). It has been shown that substrates marked with K11 chains are targeted to the 26S proteasome for degradation (Xu et al., Cell, 2009; Wu et al., PNAS, 2010). These findings, together with recent reports emphasizing K11 chain structures (Matsumoto et al., Mol Cell, 2010; Bremm et al., NSMB, 2010) and recent insights in differential ubiquitin chains and their binders in NFkappaB signaling, illustrate the interest, timing and relevance of this manuscript. The manuscript will be further improved by addressing the following questions:
Major points:
1. The authors only observed in vitro UBE2S activity when the E3 was pre-incubated with a sub-optimal amount of UbcH5B, but did not show that the catalytic activity of UBE2S is causing this effect. Furthermore, it remains unclear if the ubiquitin signals are derived from c-IAP1 autoubiquitination or unanchored ubiquitin chains (as known for UbcH5s). Dynek et al. should also include an UBE2S-binding deficient IAP RING domain and an E2-binding deficient mutant. Additionally, they could have determined the type of ubiquitin linkages in the first and second step reactions both with UbcH5A and UBE2S, using mass spectrometry.

Response:
We are thrilled that Reviewer finds our manuscript to be relevant and interesting. As requested we

tested the binding of Ube2S to E2-binding deficient and dimerization deficient c-IAP1 RING constructs. These data are presented in the new Supplemental figure 3A. We have also determined the type of ubiquitin linkages in the first and second step reactions with UbcH5a and Ube2S using mass spectrometry. Finally, we confirmed the presence of c-IAP1 in analyzed polyubiquitin bands, and in the independent experiment the presence of Ube2S in lower MW region for the lane representing UbcH5a and Ube2S in the second step reactions. These data are presented in Supplemental figure 4 and suggest that ubiquitination signals are most likely derived from c-IAP1 autoubiquitination.

2. In Figure 3, Dynek et al. stimulated cells for different time-points with CD40L, combined with knock-down of individual E2 enzymes and c-IAP1 stability as read-out. Here, the authors did not show that stimulation was effective by a read-out other than c-IAP1 stability. Also, they must show how other IAPs (interacting positively with UbcH5B in Y2H) behave, since IAPs are often found in complex with each other and could have redundant functions. Additionally, no explanation is given for the absence of any effect on c-IAP1 stability upon UBE2S knockdown.

Response:
We have used CD40-expressing cell line as a tool to examine the role of various E2 enzymes in c-IAP1 stability. We have now included new experiments showing that binding of another TNF family ligand, TWEAK to its cognate receptor causes UbcH5s dependent degradation of c-IAP1 and activation of NF-kB signaling. These data are presented in Figure 3B and C. We have also examined the fate of other IAP proteins and show that c-IAP2 and ML-IAP are not expressed in these cells while XIAP is not affected, which is expected given that XIAP does not play a role in TNF family signaling. These data are presented in Supplemental figure 5A. Regarding the lack of effect from Ube2S knockdown, we discuss this issue in the Discussion. Briefly, our data suggest that Ube2S does not play a prominent role in this aspect of c-IAP1 mediated signaling but does not preclude the possibility that Ube2S is important in other c-IAP1 mediated signaling pathways.

3. In addition, NIK ubiquitination was evaluated after over-expression of c-IAP1 and -2 in combination with E2 siRNA. Here, the authors should use c-IAP1/2 mutants in the E2 interaction interface/dimerization mutants next to wild-type E3s to strengthen their conclusions. As mentioned before, an explanation why a knockdown of UBE2S does not lead to NIK stabilization is also lacking.

Response:
We thank the Reviewer for this comment. We have now included new experiments with c-IAP1 mutated in E2 interaction interface and dimerization as requested. These data are presented in Supplemental figure 7A. Regarding the lack of effect from Ube2S knockdown, we discuss this issue in the Discussion. Briefly, our data suggest that Ube2S does not play a prominent role in this aspect of c-IAP1 mediated signaling but does not preclude the possibility that Ube2S is important in some other c-IAP1 mediated signaling events.

4. The authors investigated the E3 activity of c-IAP1 towards itself and RIP1, using over expressed RIP1, c-IAP1 and several ubiquitin mutants, followed by IPs. Again, any control, like a c-IAP1 dimerization or E2-binding mutant, is missing, just like the use of K11R, K48R and K63R mutants. The in vitro ubiquitination reaction in Figure 4B should be done also with K11R ubiquitin and the effect of UBE2S is not determined.

Response:
We thank the Reviewer for these suggestions. We have now included new experiments with c-IAP1 mutated in E2 interaction interface and dimerization as requested. These data are presented in Supplemental figure 8A. We also performed experiments with KR mutants and evaluated the effect of Ube2S in the context of K11R ubiquitin as requested. These data are presented in Supplemental figure 8C-D.

5. Furthermore, an in vitro approach was pursued, combined with MS analysis to study the type of chains generated by c-IAP1 and UbcH5A. Here, the authors should have quantified all potential types of ubiquitin chains, since UbcH5A is capable of creating mixed chains. Additionally, the concluding sentence at page 9 could only have been done if a parallel experiment with K11R and determination of the ubiquitin-acceptor lysine on RIP1 was performed.

Response:
We performed requested experiments and presented the data for the polyUb chain linkages detected as a part of Figure 4C. During the analysis we were able to measure K48 linkages, albeit at a lower level than K11 linkages. We were unable to detect K63 linkages, although others and we have shown that UbcH5 family E2 enzymes regularly generate K48, K11, and K63 linkages in vitro. Likewise, others and we have shown in the past and in this manuscript that c-IAP1 can promote K63-linked polyubiquitination of RIP1 (Mol. Cell, 2008, 30:689-700; J. Biol. Chem. 2008, 283:24295-24299). The low affinity of anti-RIP1 antibody for immunoprecitation of Ub-RIP1 when performed in the presence of urea at most provided low levels K63 chains (< 5 fmol) for analysis. Given the nature of this sample and following manual analysis of the raw mass spectrometry data, we have concluded that data do not permit us to report a value for K63 linkages in this sample. We have also performed the experiment with K11R ubiquitin mutant and present these data in Supplemental figure 8C-D. Regarding the ubiquitin-acceptor lysine on RIP1, please see our detailed answer to comment #1 from Reviewer #3 as well as data included at the end of our response letter.

6. Dynek et al. used TNFalpha stimulation to investigate the in vivo relevance of c-IAP1 in K11 chain synthesis during signaling. c-IAP1 knock out MEFs, reconstituted with wt or mutant c-IAP1 should give rise to more clearness of the direct role of c-IAP1 and K11 in signaling. The authors must explain why in Figure 5D, knock down of UbcH13 and UBE2S yield more RIP-1 ubiquitination in combination with K11R mutants and MS analysis to demonstrate K11 involvement.

Response:
We performed requested experiment with c-IAP1 knockout MEFs. These data are presented in Supplemental figure 8B. In addition, using IAP antagonist BV6 that specifically targets IAP proteins, we depleted c-IAP1 from cells and showed that K11-linked chains are absent as a consequence of c-IAP1 absence. Regarding the possibly that slightly more ubiquitination is present as a result of UbcH13 or Ube2S knockdown, it is possible that downregulation of these E2 enzymes increases the pool of available ubiquitin that could be assembled as K11-linked chains on RIP1 through the collaborative contribution of c-IAP1 and UbcH5s. To demonstrate K11 involvement, we used K11-linkage specific antibody (Matsumoto et al, Mol. Cell 2010) as evidence of K11 presence. We also performed ubiquitination reactions with K11R mutant ubiquitin and demonstrated that RIP1 can be ubiquitinated with this ubiquitin version (these data are presented in Supplemental figure 8D.). Due to the relatively low affinity of anti-RIP1 antibody for ubiquitinated RIP1 protein in immunoprecipitations, we have been unable in several attempts to obtain sufficient endogenous Ub-RIP1 for direct linkage characterization by mass spectrometry (MS). The practical limits of MS analysis indicate that limits of quantitation for individual peptides/linkages vary between 0.5-5 fmol. Note that in vitro ubiquitination of 5 g RIP1 protein, followed by IP immunoprecipitation with urea (required to dissociate Ub-proteins besides RIP) netted only ~11 fmol Ub-RIP for analysis.

Minor points:
1. The authors tested four different IAP RING-finger domains against a panel of 30 human E2 prey constructs and detected selective E2-E3 interactions. Later on, E2 interface mutants in the RINGs were generated, displaying altered E2 interactions. The authors did not show how these RING mutants interact with all other E2s within their panel. Additionally, the sequence alignment in Figure 1C showed a very conserved RING domain for c-IAP1, c-IAP2 and ML-IAP, but the authors do not discuss why these conserved RINGs display deviant E2 interaction patterns. In addition, the authors did not include UbcH10 in their screening.

Response:
We performed additional binding studies with IAP RING domain mutants and several E2 enzymes to show the specificity of these interactions. These data are presented in Supplemental figure 3A-B. Regarding the preference of IAP RING domains for particular E2 enzymes, we are not completely sure what is basis for this selectivity but future structural studies should enhance our understanding of these binding preferences. As for the comment that UbcH10 was not included in our screening, it seems that the Reviewer overlooked Supplemental Table 1 as UbcH10 was included in our initial screen.

3. The authors should mention that some E2 enzymes, like RAD6B and UBE2Q2, have close homologous that could provide redundant functions.

Response:
We thank the Reviewer for this suggestion. We have expanded our Discussion to incorporate this information.

Referee #2 (Remarks to the Author):

The ubiquitin conjugation system has shown to play crucial roles in NF-kappaB activation and various types of polyubiquitin chains including K48, K63, and linear polyubiquitin chains, have shown to be involved in the pathway. In this manuscript, the authors demonstrated the involvement of another polyubiquitin chain, the K11 chain in the NF-kappaB activation pathway using newly developed anti-K11 chain specific antibody. The authors screened E2 partners of IAP RING domains using a directed yeast two-hybrid system, and identified some novel and known E2s. Among these E2s, they focused on UbcH5s (UbcH5a, UbcH5b, and UbcH5c) as E2 partners for cIAP1 and c-IAP2. Using siRNA-mediated knockdown of UbcH5s, the authors indicated that UbcH5s are involved in degradation of c-IAP1, c-IAP2, and NIK, which are the known targets of c-IAPs. The authors also showed that c-IAP1 and UbcH5a promote K11-linked polyubiquitination of RIP1 besides K48 and K63 chains. Moreover, the authors showed that the ubiquitin binding motif of NEMO, the regulatory subunits of IkB kinase (IKK) complex, can bind to K11 di-ubiquitin in the almost same affinity as K48 and K63 di-ubiquitin and that NEMO can bind to RIP1 conjugated with K63 and/or K11 linked chains. Since it has been reported that TNF-alpha-induced IKK activation requires Ubc5s and non-K63 polyubiquitination of RIP1, it is interesting that K11-linked chain may be involved in NF-kappaB activation.
Although the data are interesting, there are a lot of caveats in this manuscript. For example, the authors showed knockdown of UbcH5s can suppress CD40L-induced RIP1 ubiquitination, but they have not shown whether UbcH5s knockdown also suppresses CD40L-induced NF-kappaB activation or not. Therefore, this manuscript cannot be included in the EMBO Journal in the present form. My comments are listed below.

Major comments
1. As pointed above, the authors have not shown that UbcH5s knockdown suppress CD40L-induced NF-kappaB activation or not using their siRNAs for UbcH5s although they showed knockdown of UbcH5s can suppress CD40L-induced RIP1 ubiquitination. Without this result, the reviewer feels that involvement of K11-linked chains in NF-kappaB activation is not sure.

Response:
We are glad that Reviewer finds our manuscript interesting. It seems that the Reviewer possibly confused different TNFR family pathways: TNFR1 does, but CD40 does not recruit RIP1 to its signaling complex, nor does it lead to RIP1 ubiquitination. Thus, we did not, or could not show that CD40L induced RIP1 ubiquitination. Instead, we have demonstrated that TNF stimulates c-IAP1 and UbcH5 dependent RIP1 ubiquitination (Figure 5).

2. K11 chains have shown to function as a degradation signal. The reviewer is curious whether K11 chains conjugated to RIP1 also functions as degradation signals as K48 chains do or not.

Response:
We performed requested experiments. These data are presented in Supplemental figures 11B and C.

3. The authors have shown that c-IAP1 can generate K11-linked chains together with UbcH5a. However, they have not examined whether c-IAP1 can generate K11 chains together with UbcH5b or UbcH5c. Although the authors have shown that siRNA mediated knockdown of UbcH5s can suppress K11 chain conjugated RIP1 (Figure 5D), they have used mixture of siRNAs targeting UbcH5a, and both UbcH5b and c. Therefore, the reviewer feels it is critical to examine this point to demonstrate the involvement of K11 chains in NF-kappaB activation.

Response:
We thank the review for this suggestion. We have performed requested ubiquitination experiments with UbcH5a, UbcH5b and ubcH5c. These data are presented in Supplemental figure 8D.

4. In the same line, it has been suggested that c-IAP1 and c-IAP2 play redundant functions. The authors showed that c-IAP1 can generate K11-linked chains in the presence of UbcH5a, but they have not examined whether c-IAP2 can generate K11 chains or not. This is another important point to be addressed to show the involvement of K11 chains in NF-kappaB activation.

Response:
We performed requested experiments with c-IAP2. These data are presented in Supplemental figures 9A-C.

5. The authors have shown that knockdown of UbcH5s suppresses degradation of c-IAP1, c-IAP2, and NIK. It might strengthen the authors' observation if introduction of UbcH5a, UbcH5b or UbcH5c, which are resistant for the siRNAs, can overcome suppression of the degradation of c-IAP1, c-IAP2, and NIK induced by the introduction of siRNAs for UbcH5s.

Response:
We performed requested experiments. These data are presented in Supplemental figure 7B.

6. In Fig3, the authors indicate that UbcH5s are involved in the degradation of c-IAP protein and its target NIK. However, this conclusion is obtained using CD40-overexpressing HKB11 cells. Is this data reproducible using primary human B cells or B cell lines expressing endogenous CD40? Human B cells can be easily knockdown by lentiviral vector. Furthermore, The reviewer is wondering if c-IAP1 degradation is inhibited by stimulations other than CD40 ligand (ex. BAFF).

Response:
The stable knockdown of UbcH5a/b/c in primary B cells could take several months. In addition, the Reviewer asks whether c-IAP1 degradation stimulated by other TNF ligands could be inhibited by the absence of UbcH5s. Thus, to address these questions we downregulated UbcH5s and stimulated cells with a different TNF family ligand, TWEAK to show the effect of UbcH5a/b/c knockdown in endogenous system. Our data show that knockdown of UbcH5 family affects c-IAP1 stability and inhibits TWEAK and TNF stimulated gene expression. These data are presented in Figure 3B-C and Supplemental figure 6.

7. It has been reported that TNF receptor activation resulted in the recruitment of not only NEMO but also TAB2 in a ubiquitination-dependent manner. It has been suggested that TAB2 binding to K63-linked chain of RIP1 activates TAK1 and IKK. Considering K11-polyubiquitinaiton of RIP1 under CD40L stimulation, it is possible that TAB2 can bind to K11-linked chain as well as NEMO. The reviewer is curious to know whether TAB2 can bind to K11-linked chain by the same method of Fig.6.

Response:
We thank the Reviewer for this interesting question. Again, we have to clarify that it is TNF, not CD40L that stimulates RIP1 ubiquitination. In addition, David Komanderís group tested the binding of TAB2 to K11-linked ubiquitin chains and reported that TAB2 does not bind K11-linked chains (Nat Struct Mol Biol. 2009 (12):1328-30).

Minor comments
1. The authors mentioned in "Introduction" that K63-linked ubiquitin chains might not be essential for TNF-alpha stimulated NF-kappaB activation (Page 3, line 16). The authors showed that RIP1 can be conjugated both K11 and K63 chains by UbcH5s, and the ubiquitin-biding domain of NEMO can bind to both K63 and K11 with almost equal affinity. If K63 chains might not be involved in NF-kappaB activation, it might be also the case with K11 chains. The reviewer recommend to correct the sentence cited above.

Response:
We have corrected this sentence to indicate that K63-linked ubiquitination is not absolutely essential for TNF stimulated NF-kB activation.

2. Fig.1; ML-IAP RING 254 is not V in Fig.1C. Furthermore, there is no sequence of ML-IAP 296 in Fig.1C. Is it correct the sequence number of ML-IAP RING in Fig.1C?

Response:
We thank the Reviewer for pointing out this mislabeling that was corrected in the revised version of our manuscript (ML-IAP has two isoforms and we made sure now that labeling is consistent throughout the manuscript).

3. It is helpful to understand the meaning of some abbreviations (ex. rxn) if the authors provide full name of the abbreviations.

Response:
We have provided explanation for this abbreviation.

Referee #3 (Remarks to the Author):

In this manuscript titled Dynek et al demonstrate that c-IAP1 and UbcH5 family of E2 enzyme UbcH5a promote K11-linked ubiquitination of RIP1, an essential molecule of TNF-induced NF-kB activation. They show that TNF-induced TNFR1-associated signaling complex includes RIP1 modified with K11-linkages. They infer that this K11 ubiquitination is mediated by c-IAPs because treatment with BV6 resulted in the loss of both c-IAP1 and modified RIP1. The authors demonstrate that ubiquitin-binding domain (UBD) of NEMO, a critical component of IKK complex involved in the activation of canonical NF-kB activation, bind in vitro to ubiquitin dimers of varying linkages including K11. Using overexpression system, association between FLAG-NEMO and RIP1 ubiquitinated with K11 linkages was shown. Recruitment of c-IAPs to the TNFR1 and their role in RIP1 polyubiquitination upon TNFa stimulation are known and the authors finding that c-IAP1 promote K11-linked ubiquitination of RIP1 is new and is important. By and large I think the data presented here support the authors' claims however, I'm not convinced that the work presented here goes far enough and I have a few specific comments that I think the authors should address.

1. The authors imply that c-IAP1 linked K11-linked ubiquitin chains are involved in TNF-induced activation of NF-kB. Direct evidence for this is needed. Results presented in figure 3 clearly show that upon CD40L stimulation, c-IAP1/UbcH5-mediated ubiquitination events promote degradation of both c-IAP1 and its substrate NIK. How can the authors rule out that in TNF-stimulated cells, RIP1 ubiquitination with K11 linkages does not lead to RIP1 degradation? Binding of NEMO to K11-linked ubiquitin chains alone does not rule out such a possibility, because results presented in figure 6C and earlier reports show that NEMO can associate with K48-linked chains, known to promote protein degradation. Identification of lysine residues on RIP1 modified by c-IAP1 with K11-linkages and characterization of importance of the lysine residues, by mutation analysis, in NF-kB activation would support the authors' claims.

Response:
We are glad that Reviewer finds our manuscript to be novel and important. The Reviewer asks whether K11 chains could lead to RIP1 degradation. We have performed experiment to address this question and present these data in Supplemental figure S11B and C. The Reviewer also suggests that identification and mutation of putative acceptor lysine on RIP1 for K11 ubiquitination would support our claims. We have tested the ubiquitination status of couple of RIP1 constructs with mutations in amino acid residues that have been reported in literature as potential acceptor lysines for RIP1 ubiquitination (Molecular Cell 22, 245ñ257, April 21, 2006). Unfortunately, none of these residues affected RIP1 ubiquitination in our assay (please see the figure below responses). Given the plasticity of ubiquitination system and the potential of multiple lysine residues to substitute for each other as acceptor sites it is uncommon for a single lysine to have a dominant role. Where mutagenesis has been successful at ablating ubiquitination, it has frequently required substitution of entire clusters of lysine residues (Mol Cell. 2006 Mar 17;21(6):737-48). The are many more unpublished examples where even combinatorial mutagenesis has been unsuccessful at ablating ubiquitination, and even reports indicating that ubiquitination can occur on non-lysine residues (Cys, also possibly Ser and Thr) when lysines are not available. Indeed, previous Mass Spectrometry characterization of RIP1 ubiquitination has indicated that numerous lysines on RIP1 may be modified by ubiquitin (Rapid Commun. Mass Spectrom. 2007;21(20):3357-64).

2. The authors conclude on page 11 that: "... these results indicate strongly that K11-linked ubiquitin chains can serve as a molecular signal for recruitment of NEMO and participate in TNF

signaling." Direct evidence under physiological conditions for participation of K11 linkages in NF-kB or TNF signaling is required. The authors should at least show association between endogenous NEMO and endogenous RIP1 with K11 linkages in TNF-stimulated cells.

Response:
We thank the Reviewer for this suggestion. We have addressed this question experimentally and shown the association of endogenous NEMO and endogenous RIP1 with K11 ubiquitin linkages in TNF-stimulated cells. These data are presented in Supplemental figure S12.

3. In order to demonstrate involvement c-IAP1 in K11-linked ubiquitination of RIP1, cells treated with the IAP antagonist BV6 were used. The authors show that treatment with BV6 blocks TNF-induced RIP1 ubiquitination and IkB degradation, suggesting inhibition of IKK activation. Interestingly, BV6 has been shown earlier to promote both canonical and non-canonical NF-kB activation (Figure 5, Cell. 2007, 131:669-81). Can the authors explain this apparent contradiction with their earlier published data. To exclude non-specific effect of BV6, data from cells deficient in both c-IAPS are needed

Response:
We are happy to provide explanation for this apparent contradiction. In our 2007 Cell publication we tested cell lines that are sensitive to induction of apoptosis by IAP antagonist BV6. In those cell lines BV6 stimulates both canonical and noncanonical NF-kB pathways. However, majority of cells are resistant to killing by IAP antagonists and pre-treatment with BV6 can deplete c-IAPs without activating these signaling pathways as others and we have shown (J Biol Chem. 2008 Sep 5;283(36):24295-9, Mol Cell. 2008 Jun 20;30(6):689-700). Therefore, usage of IAP antagonists to eliminate c-IAP proteins to study their role in signaling has been established and reported in the past. Regarding the putative non-specific effect of BV6, the specificity of this reagent was thoroughly investigated and documented in 2007 Cell publication (Cell. 2007 Nov 16;131(4):669-81). In addition, very similar agents were shown to be inactive in c-IAP knockout cells in a parallel Cell publication (Cell. 2007 Nov 16;131(4):682-93).

293T cells were transiently transfected with Flag c-IAP1, wild type or K11-only HA-tagged ubiquitin and indicated Myc RIP1 constructs. 24 hours later cells were lysed, lysates boiled in NP40 lysis buffer containing %1 SDS for 10 minutes, diluted 10-fold and immunoprecipitated with anti-Myc or anti-Flag beads. Immunoprecipitated ubiqutinated proteins were detected with anti-HA and inputs from lysates with anti-Myc, anti-Flag, or anti-Actin antibodies.

Acceptance letter 29 October 2010

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 towards publication in The EMBO Journal!

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

Referee 1 (comments to authors):

The authors have done a good work in responding to all comments raised in the

first review. Their responses are quite extensive and address my major concerns.

Referee 2 (comments to authors):

The reviewer must admit that the authors substantially addressed comments raised by the reviewer although two points listed below are not clear yet. However, the reviewer feels that this manuscript is suitable to be included in the EMBO Journal because the concept that K11 chains are involved in NF-kB signaling is new.

1. The roles of Ube2S in K11 chain formation by c-IAPs. The authors have shown that K11 chains are generated with c-IAP1and UbcH5a or c-IAP2 and UbcH5b in vitro even if they did not add Ube2S in their assay and that siRNA-mediated knockdown of Ube2S had virtually no effect on TWEAK- or TNF-a mediated NF-kB activation. Moreover, they could not find interaction between Ube2S and c-IAP2. The reviewer just feels that roles of Ube2S for generation of K11 chains by cIAPs might be very limited. So, the reviewer recommends to remove Figure 2 from this manuscript, which makes the claim of the authors more clear.
2. The role of K11 chains in NF-kB activation. The authors showed that the ubiquitin binding domain of NEMO can bind to K11 chains. But, it has not been shown whether K11 chains are indeed enough to recruit NEMO to RIP1 or not although the reviewer understand that it is very difficult to show.

